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Organization and Expression of the *Escherichia coli* K-12 *dad* Operon Encoding the Smaller Subunit of D-Amino Acid Dehydrogenase and the Catabolic Alanine Racemase

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A fragment of the *Escherichia coli* K-12 chromosome complementing the D-amino acid dehydrogenase and catabolic alanine racemase deficiency of a *dad* operon deletion mutant was cloned in a mini-Mu plasmid. The *dadA* and *dadX* genes were localized to a 3.5-kb part of the plasmid insert. The nucleotide sequence of this fragment revealed two open reading frames encoding 432- and 356-amino-acid-long proteins. We show here that they correspond to the *dadA* and *dadX* genes. The *dadA* gene can encode only the smaller of the two subunits of D-amino acid dehydrogenase. A computer search revealed the presence of a flavin adenine dinucleotide-binding motif in the N-terminal domain of the deduced DadA protein sequence. This is in agreement with biochemical data showing that the D-amino acid dehydrogenase contains flavin adenine dinucleotide in its active center. The predicted *dadX* gene product appeared to be 85% identical to a *dadB*-encoded catabolic alanine racemase of *Salmonella typhimurium*. The organization of the *dadA* and *dadX* genes confirmed our previous conclusion based on the genetic data (J. Wild, J. Hennig, M. Łobocka, W. Walczak, and T. Kłopotowski, Mol. Gen. Genet. 198:315–322, 1985) that these genes form an operon. The main transcription start points of the *dad* operon were determined by primer extension. They are preceded by a putative σ^{70} promoter sequence and two cyclic AMP-cyclic AMP receptor protein (cAMP-CRP) binding sites, one of higher and one of lower affinity to CRP. We propose that the high-affinity site, centered 59.5 bp upstream of the main transcription start point, plays a role in cAMP-CRP-mediated activation of *dad* operon expression in the absence of glucose.

Alanine in either L or D stereoisomeric form can be utilized by *Escherichia coli* as a sole source of carbon, nitrogen, and energy. The L-alanine catabolic pathway proceeds in two steps: racemization of the L isomer to D-alanine by alanine racemase and oxidative deamination of D-alanine to pyruvate and ammonia by D-amino acid dehydrogenase (43).

Of the two enzymes of alanine catabolism in *E. coli* K-12, only D-amino acid dehydrogenase has been purified to homogeneity and characterized (41). The protein is a heterodimer and contains flavin adenine dinucleotide (FAD) and nonheme iron in its active center. It has a broad specificity towards D-amino acids, of which D-alanine is the best substrate. The enzyme is membrane bound and directly linked to a respiratory chain (43). Purified D-amino acid dehydrogenase reduces ubiquinone analogs in the presence of D-alanine (42). Oxidative deamination driven by the enzyme supplies energy for an active transport (29, 30, 42).

The genetics of alanine catabolism is poorly understood. Wild and Kłopotowski (61) isolated mutants of *E. coli* K-12 unable to use either L- or D-alanine as a carbon source and lacking D-amino acid dehydrogenase activity. The mutations were mapped at the 26-min position of the *E. coli* chromosome. The identification of mutants that synthesize the temperature-sensitive enzyme allowed the authors to establish that the affected gene, designated *dadA*, is a structural gene for D-amino acid dehydrogenase. It was not clear, however, whether the *dadA* locus encodes one or both enzyme subunits. The existence of a separate gene for each subunit was suggested by independent results of Beelen et al. (4) and Franklin and Venables (14). Both groups isolated mutants, designated *alnA* and *dad*, respectively, which are unable to use D-alanine as a carbon and nitrogen source and which lack the ability to convert D-alanine into pyruvate. Because of the similar location of mutations, mapped at 1 min of *E. coli* chromosome, between the *ara* and *leu* genes, Franklin et al. (15) proposed that they affect the same gene, which the authors called *dadB*. However, Wild and Kłopotowski (61) showed normal D-amino acid dehydrogenase activity in strains containing a deletion of the *ara-leu* region of the chromosome. They also found that *dadB* mutants are defective in catabolism of pyruvate, which is an intermediate of alanine degradation. Therefore, the location of the *dadB* gene and the role of its product in D-alanine utilization need further clarification.

In our previous studies, we identified a *dadX* gene encoding the catabolic alanine racemase and mapped it distal to *dadA* (59). The *dadX* mutants are able to use D-alanine but not L-alanine as a carbon source. Genetic data indicate that the *dadA* and *dadX* genes form an operon transcribed counterclockwise from a promoter upstream of *dadA* (59). Several insertions and deletions in *dadA* exert a polar effect on the expression of *dadX*. Mutations in a regulatory region, *dadR*, preceding *dadA*, similarly affect the intracellular level of both D-amino acid dehydrogenase and catabolic alanine racemase (59, 63).

Transcription of the *dad* operon is induced by either alanine

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TABLE 1. *E. coli* K-12 strains used during this study

Strain	Genotype	Source or reference[a]
MC4100	*araD139* Δ(*argF-lac*)*205 deoC1 flb-3501 ptsF25 relA rpsl150*	10, 32
EC1094	As MC4100 but Δ(*dad-fadR*)*286 metF185 trpE6185*	59
EC1769	As EC1094 but lysogen of Mu *cts*	This study
XPh43	Δ(*argF-lac*)*U169 trp* Δ(*brnQ phoA proC phoB phoR*)*24*	21
EC1631	As MC4100 but *metF185 trpB202* λdΦ (*dadA-lac*)*1* λW	59
EC2098	As EC1631 but Δ*crp malT*::Tn*10*	T(T4) of EC2080 with EC1631
EC2126	As EC1631 but Δ*cya zie-296*::Tn*10*	T(P1) of EC2081 with EC1631
CA8445	HfrH *thi* Δ*cya* Δ*crp*	J. Beckwith
EC2080	HfrH *thi* Δ*cya* Δ*crp malT*::Tn*10*	T(P1) of MM130 with CA8445
EC2081	HfrH *thi* Δ*cya* Δ*crp zie-296*::Tn*10*	T(P1) of SK2210 with CA8445
MM130	*leuB6 his-4 rpsL136 thi-1 ara-14 lacY mtl-1 xyl tonA31 tsx-78 tsr-1 malT*::Tn*10*	J. M. Brass
SK2210	Δ(*lacZY*)*286 tsx-3 supE44 hisG4 rpsL281 xyl7 mtl-1 zie-296*::Tn*10 argH* ϕ80dIIIac Δ*lacZ95*	B. Bachman

[a] T indicates that P1*vir* (P1)- or T4GT7 (T4)-mediated transduction served to introduce the latest marker.

stereoisomer when present in the growth medium (59, 62). However, L-alanine appears to be an internal inducer since in *dadX* mutants lacking alanine racemase D-alanine does not induce *dad* operon expression (59). Franklin et al. (15) proposed that a product of the *dadQ* (*alnR*) gene, mapped at min 99 of the *E. coli* chromosome, is a positive regulator of the *dad* operon. In the *dadQ* mutant, L-alanine does not stimulate D-amino acid dehydrogenase synthesis (4).

The induced and noninduced levels of alanine catabolism enzymes are significantly lower in cells grown in the presence of glucose (14, 34, 59, 61). This regulation occurs at the transcriptional level (62).

Our report describes the structure of the *dad* operon and its predicted products. We discuss some mechanisms regulating the operon expression and present evidence for direct involvement of a cyclic AMP-cyclic AMP receptor protein (cAMP-CRP) complex in the control of *dad* transcription.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The strains of *E. coli* K-12 used in this study are listed in Table 1, except for strain JM101 (45), which served to propagate M13 phages and Bluescribe plasmid derivatives. The helper Mu *cts*62 phage was generously provided by M. Casadaban. The plasmids used in this study are shown in Table 2.

Media and growth conditions. Growth media and culture procedures were those described previously (59, 62). For induction of *dad* operon expression, media were supplemented with 10 mM L-alanine or 20 mM D-alanine when indicated. Tetracycline, ampicillin, or chloramphenicol, when needed, were added to the final concentration of 15 μg/ml, 100 μg/ml, or 50 μg/ml, respectively. TTC medium was prepared according to Bochner and Savageau (6). To differentiate between *fadR*$^+$ and *fadR* colonies, TTC medium supplemented with 5 mM decanoate (50) was used. Colonies which were unable to metabolize decanoate (*fadR*) turned red on this medium, in contrast to white *fadR*$^+$ colonies, which were able to do so. TTC media supplemented with 25 mM L- or D-alanine were employed for identification of DadA and DadX phenotypes. The *dadA dadX*$^+$ mutants unable to use either L- or D-alanine as a carbon source form white colonies on TTC medium containing either of the alanine isomers, in contrast to the red colonies of *dadA*$^+$ *dadX*$^+$ strains (61). The *dadA*$^+$ *dadX* mutants form white colonies on L-alanine- and red colonies on D-alanine-supplemented TTC media (59). MacConkey medium (37) containing 1% maltose or galactose was used to distinguish Δ*cya* or Δ*crp* mutants from the wild-type strain.

Enzyme assays. Alanine racemase and D-amino acid dehydrogenase assays were performed as described previously (59, 61). β-Galactosidase activity was assayed according to Miller (37).

Genetic techniques. P1 bacteriophage transduction and P1*vir* lysate preparation methods were described previously (37). Lysates of T4GT7 phage were obtained as described in reference 64. For T4GT7 transductions, equal amounts of recipient bacteria suspension and phage lysate were mixed,

TABLE 2. Plasmids used during this study

Plasmid	Genotype and/or relevant characteristic(s)	Source or reference
pEG109	MuII4042::*phoA proC* Cm^r	21
pHD1	MuII4042::*fadR dadA dadX* Cm^r	This study
pBR322	Ap^r Tc^r	45
pBR325	Ap^r Tc^r Cm^r	45
pBR327	Ap^r Tc^r	45
pBluescribe	Ap^r	Stratagene Co.
pHD2	5.8-kb *Hin*dIII-*Bam*HI fragment of pHD1 in pBR327	This study
pHD3	5.8-kb *Hin*dIII-*Bam*HI fragment of pHD1 in pBR325	This study
pHD4	3.3-kb *Hin*dIII-*Pvu*II fragment of pHD2 in pBR322	This study
pHD5	2.5-kb *Pvu*II-*Bam*HI fragment of pHD2 in pBR322	This study
pHD9	4.1-kb *Ava*I-*Bam*HI fragment of pHD2 in pBR322	This study
pBSD1	3.6-kb *Bgl*II-*Kpn*I fragment of pHD3 in pBluescribe	This study
pHD11	2.3-kb *Sph*I-*Kpn*I fragment of pBSD1 in pBluescribe	This study
pBSD2	0.8-kb *Eco*RV-*Bam*HI fragment of pHD3 in pBluescribe	This study

incubated for 1 h at 30°C to express the phenotype, and plated on selective media.

Plasmid and DNA techniques. Plasmid DNA isolation, restriction digestions, ligations, subclonings, and transformations were performed by following standard protocols (2, 45).

Sequencing. Plasmids pBSD1 and pBSD2 were used as a source of restriction fragments of the *dad* operon, which were subcloned into the vectors M13mp8, M13mp9, M13tg130, and M13tg131 (31, 45). M13 phage techniques were as described elsewhere (45).

DNA sequences were determined by the dideoxynucleotide chain termination method (46), using Klenow polymerase I (Amersham) or Sequenase 2.0 (U.S. Biochemical Corp.) according to the supplier's protocols. Newly synthesized DNA was labeled with α-^{35}S-dATP (Amersham). Sequencing reaction products were resolved and detected according to standard protocols (45).

Nucleic acid and deduced protein sequences were analyzed by using Genetics Computer Group (GCG) programs (University of Wisconsin Biotechnology Center, Madison). The programs TARGSEARCH and TERMINATOR (9, 40) served to search for putative promoters and factor-independent terminator sequences. Potential cAMP-CRP binding sites were found with FITCONSENSUS (GCG) by using 18 CRP-binding sites (11) to generate the consensus pattern.

Determination of the start point of *dad* transcription. The transcriptional start point of the *dad* operon was determined by primer extension with reverse transcriptase by using a modification of the method of Belfort et al. (5). Cells of MC4100 were grown up to A_{600} = 0.5 at 30°C in M63 minimal medium (37) containing 1% glycerol as the carbon source with or without 10 mM L-alanine. Each culture (15 ml) was rapidly chilled by pouring it onto a frozen solution (5 ml) of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 μg of chloramphenicol per ml. Total cellular RNA was isolated according to the modification of a hot acidic phenol method described by Hinton (24). RNA (5 to 10 μg) was incubated with 0.5 pmol of [γ-^{32}P]ATP-labeled 21-mer primer (5′-GCAACGCCTACCA CACCACTT-3′) complementary to the beginning of the DadA coding sequence in a final volume of 20 μl at 73°C for 3 min. After samples were slowly cooled at room temperature for 30 min, they were supplemented with dithiothreitol, all four deoxyribonucleotides, and RNasin (Promega) to final concentrations of 10 mM, 350 μM, and 0.2 U/μl, respectively. The reaction, catalyzed by 10 U of avian myeloblastosis virus (AMV) reverse transcriptase (Bethesda Research Laboratories), took place in a final volume of 25 μl for 90 min at 42°C and was stopped by the addition of 1 μl of 0.5 M EDTA. To remove RNA and proteins, samples were incubated at 37°C for 30 min with 0.4 mg of T1 RNase per ml and for 1 h further with 50 μg of proteinase K per ml in the presence of 0.5% sodium dodecyl sulfate in a total volume of 50 μl. After phenol-chlorophorm-isoamyl alcohol (25:24:1) extraction and ethanol precipitation, DNA was dissolved in 5 μl of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA and separated in 6% acrylamide-bisacrylamide (20:1)–7 M urea gel as described by Sambrook et al. (45). To localize positions of 3′ ends of obtained cDNA, sequencing reactions with the same primer and pHD3 plasmid DNA as a template were run on the same gel.

Assay of cAMP-CRP binding to the *dad* regulatory region. Binding of the cAMP-CRP complex to its predicted recognition regions was tested by the gel shift assay. CRP protein was a generous gift from S. Adhya and S. Ryu. Plasmid pBSD1 served as a source of restriction fragments of the *dad* regulatory region. The 309-bp *Ava*II-*Ssp*I and the 152-bp *Bst*EII-*Ssp*I fragments of pBSD1 (see Fig. 3) were separated by electrophoresis in 4% NuSieve agarose gels (Rockland). DNA was purified from gel slices by using the GeneClean (Bio 101, La Jolla, Calif.) kit. Obtained fragments were radiolabeled with [α-^{32}P]dCTP (6,000 Ci/mM; Amersham) and the Klenow fragment of DNA polymerase I (45). After labeling, the reaction mixture was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and passed twice through Sephadex G-25 spun columns (5′Prime3′, Inc., Boulder, Col.) to remove unincorporated nucleotides. Comparable amounts of each tested DNA fragment were incubated with CRP protein in 20 mM Tris-HCl buffer (pH 8.0) containing 100 mM KCl, 2 mM dithiothreitol, 10 mM $MgCl_2$, 0.1 mM cAMP, bovine serum albumin (0.1 mg/ml), poly(dA-dC) (1 μg/ml), and 5% glycerol for 10 min at 37°C. After incubation, the mixture was loaded on a running 5% polyacrylamide gel (acrylamide to *N,N′*-methylenebisacrylamide weight ratio, 38:1) containing 0.1 mM cAMP and electrophoresed in 45 mM Tris-borate–1 mM EDTA buffer (pH 8.3).

Nucleotide sequence accession number. The DNA sequence reported here is available in GenBank under accession number L02948.

RESULTS

In vivo cloning of the *dad* operon. To clone the *E. coli dad* operon, we employed a mini-Mu bacteriophage as described by Groisman and Casadaban (21). This cloning system allows preparation of bacterial gene libraries in the form of phage lysates. Furthermore, specific features of the mini-Mu phage employed permit recovery of phages carrying bacterial DNA inserts as multicopy plasmids.

Strain XPh43, which contains the wild-type *dad* operon and is lysogenic for Mu *cts*, was transformed with mini-Mu plasmid pEG109. A phage lysate prepared on one of the transformants was used to transduce the EC1769 recipient carrying a deletion of the *dad* region. Among selected chloramphenicol-resistant colonies, *dad*$^+$ transductants able to grow on minimal medium containing L-alanine as the sole source of carbon appeared at a frequency of 2×10^{-3}. One of the *dad*$^+$ transductants served as a source of the pHD1 plasmid carrying a 7.8-kb bacterial DNA fragment inserted into mini-Mu DNA. The pHD1 plasmid complemented the Δ(*dad-fadR*)*286* strain, restoring the activity of both D-amino acid dehydrogenase and alanine racemase (Table 3). It also restored the FadR$^+$ phenotype of this strain, indicating that genes constituting the *dad* operon as well as the closely linked *fadR* gene (59) were cloned. Synthesis of D-amino acid dehydrogenase and catabolic alanine racemase expressed from the pHD1 plasmid was inducible by L-alanine, as is the synthesis of enzymes expressed from the chromosomal *dad* operon (Table 3).

The pHD2 plasmid containing the 5.8-kb *Hin*dIII-*Bam*HI fragment of the pHD1 chromosomal insert complemented the DadA$^-$ and DadX$^-$ but not FadR$^-$ phenotype of the Δ(*dad-fadR*)*286* strain. To localize the *dad* genes within the insert of pHD2, plasmids carrying its different restriction fragments were constructed and tested for complementation of the DadA$^-$ DadX$^-$ phenotype of EC1094 (Fig. 1). In the presence of the pHD9 plasmid carrying the 4.1-kb *Eco*RV-*Bam*HI fragment, strain EC1094 became DadA$^+$ DadX$^+$, implying that both genes were present and expressed within the cloned insert. Further complementation analysis suggested that the 5′ end of the *dadA* gene is located between the *Sph*I and *Pvu*II sites, as indicated by the fact that pHD5 carrying the insert starting at the *Pvu*II site did not restore the DadA$^+$ phenotype. It also did not complement the alanine racemase deficiency of EC1094. The only plasmid restoring alanine racemase activity

TABLE 3. Restoration of D-amino acid dehydrogenase and catabolic alanine racemase activities in a Δ(*dad-fadR*)*286* strain carrying the pHD1 plasmid[a]

E. coli strain/plasmid	Relevant genotype: Chromosome	Relevant genotype: Plasmid	Enzyme activity[b]: D-Amino acid dehydrogenase −	D-Amino acid dehydrogenase +	Alanine racemase −	Alanine racemase +
XPh43/pEG109	*dadA*$^+$ *dadX*$^+$	*dadA dadX*	6	72	9	60
EC1769/pEG109	Δ(*dad-fadR*)*286*	*dadA dadX*	0	0	5[c]	7[c]
EC1769/pHD1	Δ(*dad-fadR*)*286*	*dadA*$^+$ *dadX*$^+$	35	68	35	115

[a] Bacteria were grown for 18 h in minimal medium supplemented with 0.5% glucose as a carbon source. L-Alanine was added to induce enzyme synthesis, when indicated, to a final concentration of 10 mM.

[b] The specific activity of each enzyme is expressed in nanomoles of product (pyruvate or D-alanine) formed per minute per milligram (dry weight) of cells in the presence (+) or absence (−) of L-alanine. Each result is an average from at least three independent experiments.

[c] The residual alanine racemase activity in the *dadA dadX* strain represents the activity of a constitutively expressed biosynthetic isozyme encoded by the unlinked *alr* gene (59).

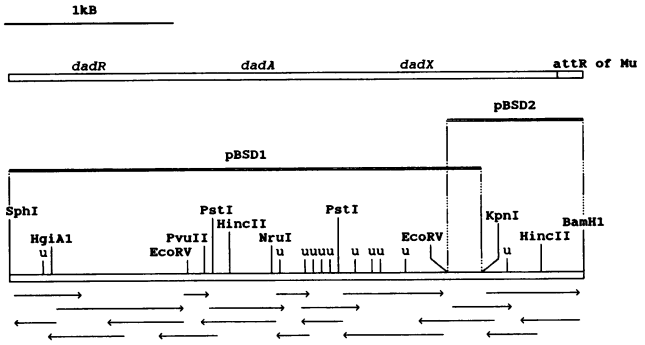

FIG. 2. Restriction map of the 3.252-kb *Sph*I-*Bam*HI fragment of the pHD2 plasmid showing the sequencing strategy of the *dad* operon. u, recognition sites for *Sau*3A. Chromosomal inserts of pBSD1 and pBSD2 plasmids used as a source of DNA for cloning into M13 phages are shown as double lines. Light single lines with arrowheads indicate the positions and extents of sequencing. The locations of genes in the sequenced fragment are indicated above the restriction map.

was pHD9 (Fig. 1). This suggests that the 3′ end of the *dadX* gene resides within the *Kpn*I-*Bam*HI fragment. The inability of the pHD5 plasmid, which contains the 3′ end of the *dadX* gene but does not contain the 5′ end of the *dadA* gene, to complement the DadX$^-$ phenotype indicates that the *dadA* and *dadX* genes comprise an operon with the promoter proximal to the *dadA* gene.

Nucleotide sequence of the *dad* operon. To determine the structure of the *dad* operon and its putative regulatory region, an *Sph*I-*Bam*HI fragment of pHD2 complementing both *dadA* and *dadX* mutations was sequenced. The strategy of sequencing, using as templates restriction fragments of *dad* cloned into different M13 vectors, is shown in Fig. 2. The complete nucleotide sequence of both strands was determined. The sequence (Fig. 3) starts at the *Sph*I site (nucleotide [nt] 1) and ends at the junction of chromosomal DNA and DNA of the mini-Mu plasmid used for in vivo cloning (nt 3262). Sequence analysis revealed the presence of two long open reading frames (ORFs), directed from the 5′ to the 3′ end and positioned in tandem. According to the results of the complementation analysis (Fig. 1), they should correspond to the *dadA* and *dadX* genes. A computer search (44) indicated ATG codons at positions 526 and 1834 as the most probable translation initiation sites inside the first (*dadA*) and the second (*dadX*) ORF, respectively. Immediately upstream of both ATG codons are regions homologous to a Shine-Dalgarno sequence, which

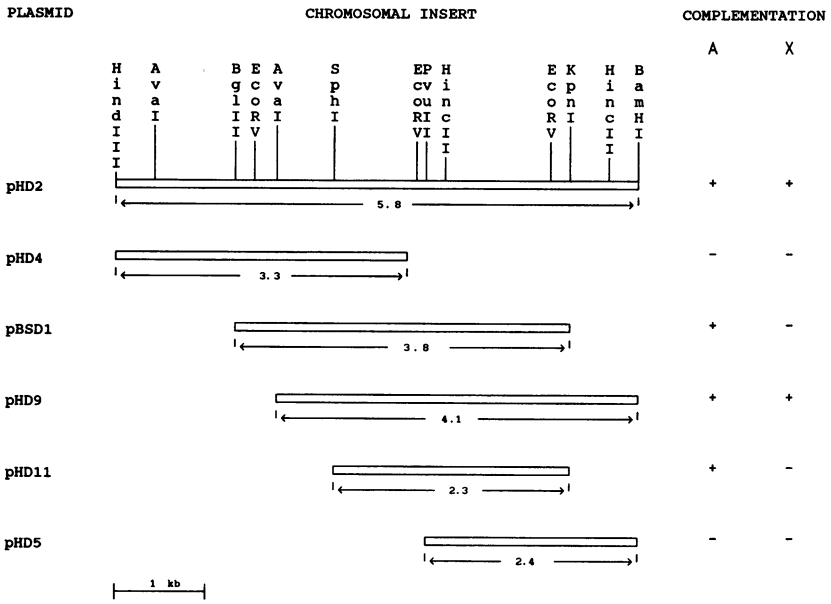

FIG. 1. Restriction map and complementation analysis of the cloned chromosomal fragment that complements the D-amino acid dehydrogenase and the alanine racemase deficiency of a Δ(*dad-fadR*)*286* strain. Designations of plasmids are given on the left of the schematic illustration of each insert. The length of each cloned insert (in kilobases) is shown. The ability of each plasmid to restore the DadA (A) and/or DadX (X) phenotype of the EC1094 strain, as measured by the assay of D-amino acid dehydrogenase and alanine racemase activities, is shown on the right.

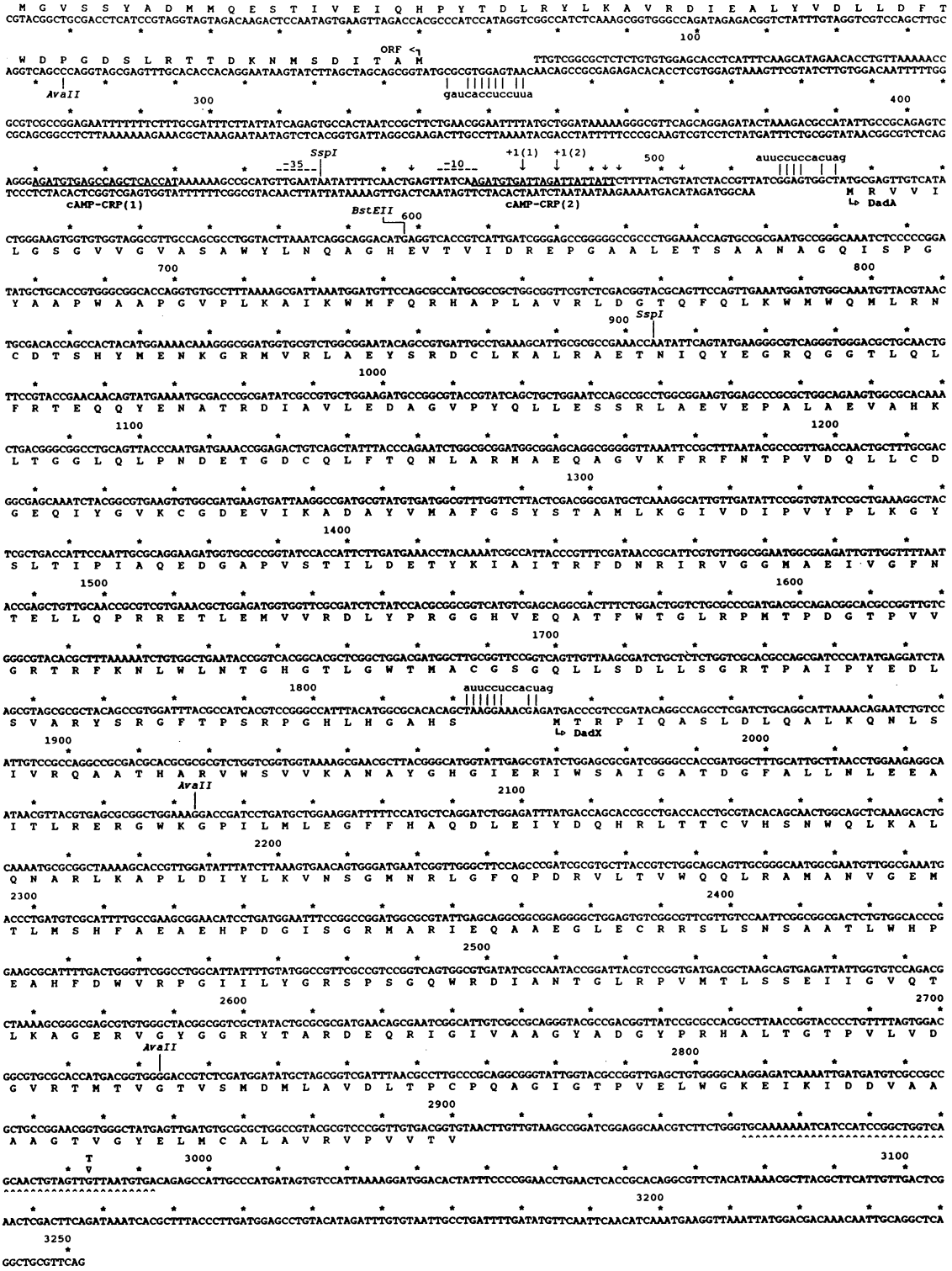

FIG. 3. Nucleotide sequence of the *dad* operon and deduced amino acid sequences of its protein products. The nucleotide sequence starts at the *Sph*I site of the pHD2 plasmid insert and ends at a junction of the DNA of *E. coli* and the mini-Mu vector used for cloning. Regions of homology with the 3′ end of 16S rRNA (lowercase letters) corresponding to ribosome binding sites are shown in front of each protein coding sequence. Transcription initiation sites are marked by arrows. Position +1(1) corresponds to the beginning of the major transcript in L-alanine-induced cells, and position +1(2) corresponds to the major transcript in noninduced cells. Dashed lines above the sequence indicate −10 and −35 regions of the putative *dad* promoter. Sequences resembling the consensus sequence for CRP-binding sites are underlined. The predicted position of the 3′ end of the *dad* transcript is marked with an arrowhead. The DNA segment labeled by underlining with carets shows a region of homology with known factor-independent transcription termination sites in *E. coli*.

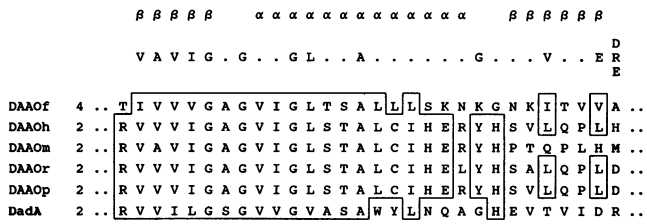

FIG. 4. Comparison of the amino acid sequences of FAD-binding domains of D-amino acid oxidases (DAAOf, *Fusarium solani* [26]; DAAOh, human [39]; DAAOm, mouse [52]; DAAOr, rabbit [38]; DAAOp, pig [16]) with the N terminus of the predicted DadA protein. Identical or functionally similar residues found in at least four sequences are boxed. Conservative replacements allowed are I/L/V, T/S, and A/S. Well-conserved residues of FAD-binding protein domains and the conserved secondary structure βαβ are shown above the sequence (as proposed by Schultz [48]).

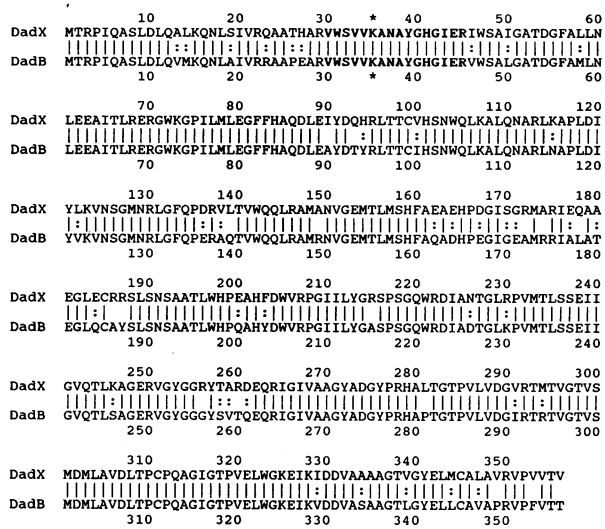

FIG. 5. Similarity of the amino acid sequence of the *dadX* gene product of E. coli K-12 (DadX) and the catabolic alanine racemase of *S. typhimurium* encoded by the *dadB* gene (DadB) (56). Vertical lines indicate identical residues, and colons indicate conservative changes. Boldface letters show amino acids of the active center with the lysine residue that interacts directly with pyridoxal phosphate (marked by an asterisk).

serves as a prokaryotic ribosome binding site (49) (Fig. 3). The initiation codon of *dadX* lies 9 bp beyond the termination codon of *dadA*, which supports the results of complementation analysis showing that there is no separate promoter for *dadX*. The *dadA* and *dadX* genes seem to be the only structural genes of the *dad* operon. There is no ORF within a 100-bp fragment of DNA downstream of *dadX*. Instead, the computer search revealed the presence of a putative factor-independent transcription terminator sequence following *dadX* (Fig. 3). The G at position 2983 is predicted to be the 3′ end of the *dad* operon mRNA.

Previous genetic studies (59) identified a *dadR* gene as a *cis*-acting regulatory region of the *dad* operon. Wild et al. (63) proposed that it contains promotor and operator sequences of *dad*. The DNA fragment of about 260 bp preceding *dadA* should correspond to *dadR*. It does not contain any putative protein-coding regions. Interestingly, it separates the ORF identified as *dadA* from an ORF oriented in the opposite direction. Therefore, the *dadR* region may contain promoter and regulatory sequences of both the *dad* operon and the unidentified gene.

Further analysis of sequences upstream of *dadR* (data not shown) indicates that the new putative gene may encode a protein of 474 amino acids (aa). Its translation should start from ATG codon at nt 196 of the sequence (Fig. 3), which is preceded by a fragment highly homologous to the 3′ end of 16S RNA. This fragment may encode a ribosome binding site in mRNA.

The *dadA* gene can encode the smaller subunit of D-amino acid dehydrogenase. Purified D-amino acid dehydrogenase is composed of two subunits, with molecular masses of 45 and 55 kDa (41). The deduced length of the *dadA* gene product is 432 aa, which corresponds to a 47.6-kDa protein. Therefore, the dadA locus has a potential to encode the smaller subunit of D-amino acid dehydrogenase. Comparison of its predicted amino acid sequence with sequences obtained from the SWISSPROT and PIR data bases revealed a protein of unknown function of *Pseudomonas aeruginosa* encoded by an ORFZ in the *pilRS* region (GenBank accession number, Z12154) as the best matching sequence. A known C-terminal fragment of this protein at 106 aa is 40% identical with the C-terminal fragment of DadA. The N terminus of DadA appeared to be similar to FAD-binding domains of various proteins (Fig. 4). Strikingly high similarity was found between the N-terminal part of deduced DadA protein and FAD-binding domains of D-amino acid oxidases (Fig. 4). A hydrophobicity plot of DadA, obtained by the Kyte and Doolittle method (33), indicated that the N terminus is a strongly hydrophobic region (data not shown).

The *dadX* gene encodes the catabolic alanine racemase. The second ORF within the *dad* operon (Fig. 3, from nt 1834 to 2901), designated *dadX*, must correspond to the catabolic alanine racemase gene. It can encode a 356-aa-long protein with a molecular mass of 39.0 kDa. The predicted product of *dadX* is 85% identical to the catabolic alanine racemase of *Salmonella typhimurium* encoded by the *dadB* gene (56) (Fig. 5) and about 40% identical to the anabolic alanine racemase of *S. typhimurium* (18) and the alanine racemase of *Bacillus subtilis* (13, 53) (data not shown). By homology, we found that the active center of the *E. coli* catabolic alanine racemase is located close to its N terminus at the same position as in the *S. typhimurium* DadB protein (Fig. 5) (3). As in other alanine racemases, it contains a lysine residue that interacts directly with pyridoxal phosphate (22). Galactos and Walsh (17) have shown that both alanine racemases of *S. typhimurium*, catabolic and anabolic, have a two-domain structure. The domains are linked by a Gly-rich hinge, which is essential for catalytic efficiency of these enzymes. The sequence of the hinge region is conserved in the *dadX* gene product (Fig. 5, aa 254 to 259), as is shown by comparison with DadB.

Mapping a 5′ end of the *dad* operon transcript. A computer search for putative promoters upstream of *dadA* revealed several regions with significant similarity to known *E. coli* σ^{70} promoter sequences. To determine which of them plays a major role in *dad* expression, we mapped the 5′ ends of the *dad*-encoded mRNA by primer extension, using reverse transcriptase (Fig. 6). We employed mRNA isolated from cells expressing the *dad* operon at the noninduced level or after induction with L-alanine. The main product, 87 nt long, which appeared as a strong band when mRNA from induced cells was used, was barely visible when mRNA from uninduced cells was used. It identifies the major transcription start site functioning upon induction at nt 480 of the sequenced DNA fragment.

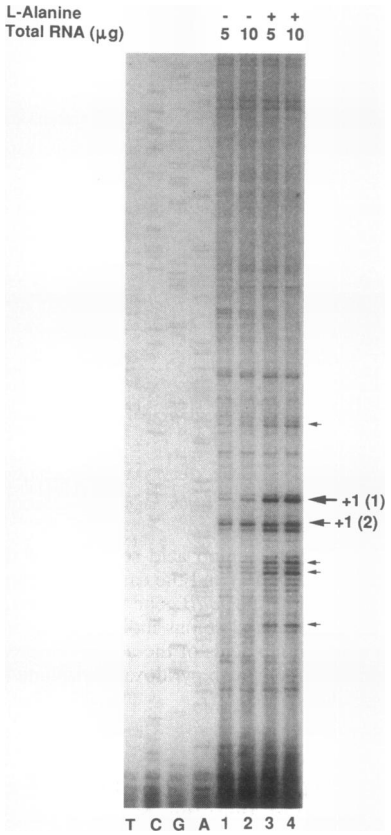

FIG. 6. Location of the transcription start points of the *dad* operon by the primer extension method. Lanes 1 to 4 show the products of the primer extension using 5 and 10 μg of total RNA from *E. coli* MC4100 growing in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 10 mM L-alanine. The sequence of [γ-^{32}P]ATP-labeled primer complementary to a region in *dadA* downstream from the transcriptional start was 5'GCAAGCCCTACCACACCACTT. The sizing ladder, synthesized with the same primer by using pHD3 as the template in dideoxy sequencing reactions, depicts the noncoding strand in lanes marked T, C, G, and A.

Consensus:	AA - TGTGA ------ TCACA - T	
		Mismatches
dadR(1)	AG A TGTGA GCCAGC TCACC A T	2
dadR(2)	AG A TGTGA TTAGAT TATTA T T	4
araB	AA TGTGT GCGCCG TGCAA A T	3
araE	AA T TGGAA TATCCA TCACA T A	5
cat	AA A TGAGA CGTTGA TCGGC A C	5
crp	TA A TGTGA CGTCCT TTGCA T A	3
crp (2)	GA A GGCGA CCTGGG TCATG C T	6
deoC	AA T TGTGA TGTGTA TCGAA G T	3
deoC (2)	TA A TGCGA TCTGGG TCAAA T A	3
gal	AA G TGTGA CATGGA ATAAA T T	3
ilvB	AA T TGAGG GGTTGA TCACG T T	4
lac	TA A TGTGA GTTAGC TCACT C A	3
lac (2)	AA T TGTGA GCGGAT AACAA T T	4
malT	AA T TGTGA CACAGT GCAAA T T	2
ompA	AA G TGTGA ACTCCG TCAGG C A	2
tna	AA A TGTGA ATCGAA TCACA A T	1

FIG. 7. Comparison of putative CRP-binding sites in the promoter-operator region of the *dad* operon [*dadR*(1) and *dadR*(2), the sequences underlined and labeled cAMP-CRP(1) and cAMP-CRP(2), respectively, in Fig. 3] with the consensus sequence of CRP-binding sites and regions interacting with cAMP-CRP complex found in other operons (11, 12).

Surprisingly, the major band detected when mRNA from uninduced cells was used corresponds to a product 5 bp shorter. This band is significantly enhanced with mRNA from induced cells, indicating that it represents an alternative transcript of the *dad* operon. Although it is the main alternative product of the primer extension experiment, it is not the only one. Weaker bands which are enhanced with mRNA from induced cells and correspond to one longer fragment and a few shorter DNA fragments may represent other minor products of *dad* transcription. They can be clearly distinguished from other weak bands of the background that have similar intensity independently of the source of mRNA used. The latter most likely represent products of nonspecific priming.

Characterization of the *dad* operon promoter-operator region. Immediately upstream of the major transcription start point of the *dad* operon, we found a putative σ^{70} promoter sequence (Fig. 3) with a calculated (40) homology to the consensus sequence of 49.1%. It is close to the homology to the consensus sequence of *lacP1* (49.7%), calculated by the same method, and suggests that the main *dad* transcript initiates from a moderately strong promoter.

The presence of a few minor primer extension products obtained when the *dad* mRNA was used may indicate the existence of several additional transcription start sites for this operon. We found that each of these putative additional starts is preceded by a sequence significantly homologous to the consensus for σ^{70} promoters (data not shown), and therefore we cannot exclude the possibility of their utilization.

Analysis of a promoter region of the *dad* operon revealed two regions highly homologous to cAMP-CRP binding sites (Fig. 3 and 7). The one with higher homology is located 59.5 bp (counting from the center of symmetry) upstream of the major transcription start site of the *dad* operon. The second one partially overlaps the −10 region of the predicted *dad* promoter.

cAMP-CRP is involved in the regulation of *dad* transcription in vivo. It was shown previously (59) that the transcription of the *dad* operon is repressed in cells grown in the presence of glucose. To test whether this effect is mediated by cAMP-CRP, we measured the activity of β-galactosidase in a strain containing the λdϕ (*dadA'-lacZ*)1 transcriptional fusion and its Δ*cya* and Δ*crp* derivatives (Table 4). In cells of the wild-type strain grown in rich medium without glucose, the enzyme activity was almost four times higher then in cells from glucose-containing medium. In both Δ*crp* and Δ*cya* mutants, the derepression of β-galactosidase synthesis in the absence of glucose did not

TABLE 4. Expression of Φ(*dadA'-lacZ*)*1* fusion in Δ*crp* and Δ*cya* mutants[a]

E. coli strain	Relevant genotype	Sp act of β-galactosidase[b]	
		−	+
EC1631	*cya*$^+$ *crp*$^+$	820	236
EC2098	*cya*$^+$ Δ*crp*	185	214
EC2126	Δ*cya crp*$^+$	213	152

[a] Bacteria for enzyme assays were grown for 4.5 h in Luria-Bertani medium supplemented with 0.5% glucose, when indicated.

[b] The specific activity of β-galactosidase in the presence (+) or absence (−) of glucose is expressed in Miller units (48). Each result is an average from at least three independent experiments.

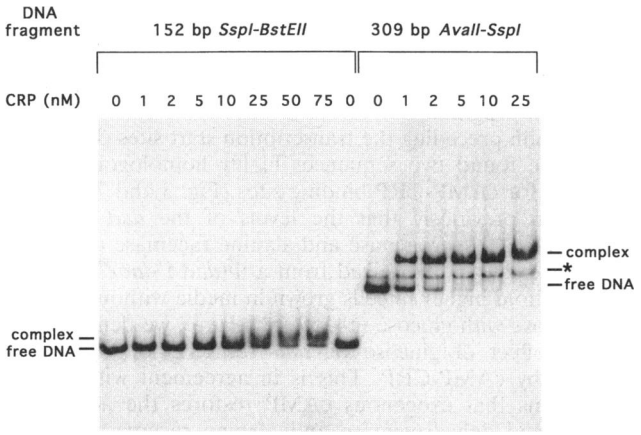

FIG. 8. Binding of cAMP-CRP complex to the ^{32}P end-labeled fragments of the *dad* regulatory region, as measured by gel shift assay. The assay conditions are described in Materials and Methods. CRP levels are given as concentrations of dimer. cAMP at a concentration of 0.1 mM was present in all samples, in a gel and in running buffer. No mobility shift was observed in the absence of cAMP (data not shown). The band marked with an asterisk represents a contaminating fragment of pBSD1 plasmid which was not fully removed during the purification of the desired DNA fragment.

occur, indicating that the effect of glucose on *dad* transcription is mediated by cAMP-CRP.

CRP binds to DNA fragments of the *dad* regulatory region. To test whether a cAMP-CRP complex might regulate the operon transcription by direct interaction with its putative recognition sites in the regulatory region of *dad*, we isolated 309-bp *Ava*II-*Ssp*I and 152-bp *Ssp*I-*Bst*EII fragments of the *dad* regulatory region containing each of the two identified sites separately. A gel shift assay experiment with purified CRP protein and cAMP demonstrated that both fragments can bind the cAMP-CRP complex, although with different affinities (Fig. 8). The 309-bp *Ava*II-*Ssp*I fragment, containing the site which best matches the consensus sequence, bound the complex at a CRP concentration of as low as 1 nM. Binding of the complex to the 152-bp *Ssp*I-*Bst*EII fragment containing the site less similar to the consensus sequence was readily detectable at a concentration of 25 nM CRP or higher. For both fragments, we observed only one shifted DNA band indicating (in agreement with the sequence analysis data) that each of the tested regions contains a single CRP-binding site. Of special notice is a small difference between the extent to which the presence of the cAMP-CRP complex shifts the 152-bp *Ssp*I-*Bst*EII fragment and the extent to which the complex shifts the *Ava*II-*Ssp*I fragment. This can be explained by the different locations of binding sites within the fragments, since binding of cAMP-CRP complex bends DNA, and by the difference in their affinities to cAMP-CRP, which in turn influences bending (19).

DISCUSSION

Genetic studies of alanine catabolism in *E. coli* K-12 identified the *dadA* and *dadX* genes as coding for enzymes of this pathway: the D-amino acid dehydrogenase and the catabolic alanine racemase. They form an operon and are located at 26 min of the *E. coli* map (59, 61). For this study of the structure and regulation of both genes, we cloned and sequenced a DNA fragment complementing chromosomal deletion of the *dad* operon.

It appeared that *dadA* can encode only the smaller of two subunits of the D-amino acid dehydrogenase. The predicted molecular mass of its product (47 kDa) corresponds well to the mass of the smaller enzyme subunit, estimated from analysis of the purified protein as 45 kDa (41). The presence of an FAD-binding motif at the N-terminal end of DadA is in agreement with biochemical data (41) showing that the native D-amino acid dehydrogenase contains FAD in its active center. The FAD-binding domain of DadA in its sequence and location within the protein resembles those found in eukaryotic D-amino acid oxidases, enzymes with similar substrate specificities for amino acids. This indicates an evolutionary relationship. In D-amino acid oxidases, the FAD-binding domain is proposed to be a site anchoring the protein to membrane structures (16). The high hydrophobicity of this domain in DadA implies a similar role. This is consistent with the fact that the native D-amino acid dehydrogenase is bound by strongly hydrophobic interactions to the inner membrane (27, 28, 42).

Our results excluded the possibility that the *dad* operon could encode the second subunit of D-amino acid dehydrogenase. The smallest fragment complementing the deficiency of D-amino acid dehydrogenase in a Δ(*dad-fadR*)*286* deletion strain (Fig. 1, insert of pHD11 plasmid) is only 2.3 kb long and includes no complete ORF encoding a 55-kDa protein. Besides, analysis in a maxicell system of bacterial proteins expressed from the insert of the pHD9 plasmid containing the entire *dad* operon revealed only two products, with molecular masses of 44 and 37 kDa, corresponding to DadA and DadX (23).

The *dadX* gene encoding the catabolic alanine racemase corresponds to a second ORF of the *dad* operon. The 85% identity of DadX protein to DadB catabolic alanine racemase of *S. typhimurium* confirms a homology previously proposed because of similar function and location of genes (57, 59). The active center of DadX and the Gly-rich hinge known to be important for the catalytic efficiency of alanine racemases have the same structure as in other enzymes of this group and are located at the same position as in DadB (18, 22).

Recently, Thornberry et al. (54) purified to homogeneity the alanine racemase from *E. coli* B cells grown in minimal medium with L-alanine as the sole source of carbon. It had been shown previously for *E. coli* K-12 that when cells were grown in the presence of L-alanine the *dadX* gene product comprised more than 95% of overall cellular alanine racemase activity (59). This suggests that Thornberry's enzyme is a homolog of DadX in *E. coli* B. Indeed, proteolytic digestion of the enzyme with trypsin released a hexapeptide, -Val-Gly-Tyr-Gly-Gly-Arg- (54), identical to that found in position 251 to 256 of DadX (Fig. 5). On the other hand, the estimated molecular mass of alanine racemase isolated from *E. coli* B, 50 kDa (54), is significantly higher than the predicted molecular mass of DadX (39 kDa). Therefore, the identities of these two enzymes need further verification.

The sequence analysis of the *dad* operon and the results of our complementation experiments confirmed previous genetic evidence (59) that the *dadA* and *dadX* genes are transcribed together from a promoter proximal to *dadA*. The fact that the *dadA* and *dadX* genes form one transcriptional unit might suggest an organization of alanine catabolism genes in *E. coli* K-12 different from that of their homologs *dadA* and *dadB* in the closely related *S. typhimurium*. Wasserman et al. (57) cloned a DNA fragment of the latter bacterium complementing *dadB* but not *dadA* mutations. Expression of alanine racemase from this fragment was inducible by L-alanine. These data enabled the authors to conclude that in *S. typhimurium* the *dadB* and *dadA* genes are transcribed from separate

promoters and that *dadB* does not belong to the same operon as *dadA*. We suggest, instead, that the 4-kb fragment of bacterial DNA cloned by Wasserman et al. could contain a mutated *dadA* gene with its unaffected promoter-operator region. The *dadB* gene present in this fragment is 1,068 nt long, and its 3′ end overlaps the end of the insert (56). Therefore, the transcript could include almost 3 kb of message upstream of *dadB*, which is sufficient to express *dadA* in *E. coli*. Such a possibility is supported by the fact that the published sequence of 60 bp upstream of *dadB* can encode a C-terminal end of a protein which is 65% identical to the C-terminal end of *E. coli* K-12 DadA. Our difficulties in transforming some *E. coli* K-12 strains with multicopy $dadA^+$ plasmids suggest that selection for *dadA* plasmid mutants may occur in particular hosts. Perhaps the overproduction of D-amino acid dehydrogenase in those strains could lead to the lethal decrease in an intracellular pool of D-alanine, an amino acid that is required for cell wall biosynthesis in gram-negative bacteria (55). Lower levels of L-alanine-mediated induction of the *dad* operon enzymes expressed from the multicopy plasmid in a strain we used for cloning, compared with levels of the induction of the enzymes expressed from a chromosome (Table 3), suggest that in *E. coli* there is a mechanism of negative regulation preventing D-amino acid dehydrogenase overproduction. As the extent of induction of D-amino acid dehydrogenase expressed from multicopy $dadA^+$ *dadX* plasmids is similar to the extent of induction of enzyme encoded by chromosomal genes (data not shown), we think that the pool of the intracellular inducer, L-alanine (59), is limiting and can account for the observed effect. The limitation of intracellular L-alanine concentration may occur in strains with an excess of alanine racemase when the use of D-alanine by the overexpressed D-amino acid dehydrogenase moves the equilibrium of the racemization reaction strongly toward the D-isomer production.

Mapping of the 5′ end of the *dad* operon transcript showed that mRNA synthesis is initiated from at least two sites. The main start site upon induction by L-alanine is located 41 bp upstream of the DadA coding sequence, and it is preceded by a moderately strong promoter. The second, located 5 bp closer to *dadA*, predominates in noninduced cells. The presence of a few other minor transcription start sites is suggested by additional products of primer extension obtained with *dad* operon mRNA (Fig. 6). Although this possibility requires further confirmation, it is supported by the fact that sequences homologous to σ^{70} promoters precede each of those putative start sites. The existence of multiple *dad* promoters would be consistent with a complex regulation of this operon expression. It was shown to be activated by alanine (59, 61) and cAMP (14). Recent studies in our and other laboratories have expanded the list of *dad* regulatory factors with leucine (35), anaerobic activator protein FNR (35a), and histonelike protein IHF (36).

Previous genetic studies established that the *dadR* locus located between the *fadR* and *dadA* genes is a regulatory region of the *dad* operon (63). Several mutations of this region caused increased activity of both D-amino acid dehydrogenase and catabolic alanine racemase (58, 59, 60, 63). One of them, *dadR1*, was studied in a strain carrying a Φ (*dadA′-lacZ*$^+$) fusion (62). It was found to have no *trans* effect on β-galactosidase expression (63). This suggests that the *dadR* locus corresponds to the promoter-operator region of the *dad* operon and lies within the 260-bp-long DNA fragment between *dadA* and the ORF encoding an unidentified protein. The latter is the only structural gene separating the *dad* operon from *fadR* (data not shown). Its presence in a multicopy plasmid does not influence either induced or uninduced expression of β-galactosidase from Φ(*dadA′-lacZ*$^+$) chromosomal fusion (23). Thus, the possibility that it encodes a *trans*-acting *dad* regulatory factor seems to be unlikely. Our studies to identify the function of the product of this ORF are currently in progress.

In a region preceding the transcription start sites of the *dad* operon, we found two sequences highly homologous to the consensus for cAMP-CRP binding sites (Fig. 3 and 7) (12). It was shown previously that the levels of the *dad* enzymes D-amino acid dehydrogenase and alanine racemase as well as the β-galactosidase expressed from a Φ(*dadA′-lacZ*$^+$) fusion are severalfold higher in cells grown in media without glucose than in those with glucose (14, 59, 61). Here, we demonstrate that the effect of glucose on *dad* transcription in vivo is mediated by cAMP-CRP. This is in agreement with earlier observations that exogenous cAMP restores the activity of D-amino acid dehydrogenase and alanine racemase in cells grown with glucose to 80% of the level observed in the absence of glucose (14). In vitro binding of cAMP-CRP to fragments of the *dad* regulatory region containing either of its recognition sequences separately suggests that both identified sequences are involved in the cAMP-CRP-mediated regulation of *dad* transcription in vivo. Of the two sites, the one with higher affinity to the complex and the better match to the consensus sequence (Fig. 3 and 7) is centered at 59.5 bp upstream from the main transcription start point of *dad* mRNA. This resembles the position of a cAMP-CRP binding region of the *E. coli* *lac* promoter, −61.5 (47, 51), which was found to be one of two (the other being at −41.5) that are optimal for the contact of CRP with RNA polymerase that is required for CRP-mediated transcription activation (20). Numerous studies have shown that cAMP-CRP when bound 59.5 bp upstream of the promoter can bring about the same or a slightly weaker activation of transcription than when positioned at −60.5 or −61.5 bp (1, 8, 20). This implies that the increase of *dad* transcription in vivo in cells grown without glucose could be mediated by the interaction of the complex with this site.

The role of the second identified cAMP-CRP binding site in *dad* regulation is unclear. It partially overlaps the −10 sequence of the *dad* promoter and the transcription initiation region, implying that interaction of the complex with this site might interfere with transcription as in the case of some promoters repressed by cAMP-CRP (for a review, see reference 7). Perhaps the net expression of the *dad* operon reflects a sum of positive and negative effects of cAMP-CRP complex binding on the activity of its promoters, as is the case with the *gal* operon (25).

Although for *E. coli* there are no genetic data indicating the presence of cAMP-CRP binding sites in the *dad* regulatory region, such data were obtained for the related bacterium *S. typhimurium*. Wild and Kłopotowski (60) isolated mutants of this bacterium in which the synthesis of D-amino acid dehydrogenase did not undergo catabolite repression, although it remained inducible by alanine. The mutations were mapped upstream of *dadA* in a region corresponding approximately to the *dadR* locus of *E. coli*, i.e., exactly where two cAMP-CRP binding sites are located.

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REFERENCES

1. **Aiba, H., A. Hanamura, and T. Tobe.** 1989. Semisynthetic promoters activated by the cyclic AMP receptor protein of *E. coli*. Gene **85**:91–97.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. Greene Publishing Associates and John Wiley & Sons, Inc., New York.
3. **Badet, B., D. Roise, and C. T. Walsh.** 1984. Inactivation of the *dadB Salmonella typhimurium* alanine racemase by D- and L-isomers of β-substituted alanines: kinetics, stoichiometry, active site peptide sequencing, and reaction mechanism. Biochemistry **23**:5188–5194.
4. **Beelen, R. H. J., A. M. Feldman, and H. J. W. Wijsman.** 1973. A regulatory gene and a structural gene for alaninase in *Escherichia coli*. Mol. Gen. Genet. **121**:369–374.
5. **Belfort, M., J. Pedersen-Lane, J. West, D. Ehrenman, K. Maley, G. Chu, and F. Maley.** 1985. Processing of the intron containing thymidylate syntase (td) gene of phage T4 is at the RNA level. Cell **41**:375–382.
6. **Bochner, B. R., and M. A. Savageau.** 1977. Generalized indicator plate for genetic, metabolic, and taxonomic studies with microorganisms. Appl. Environ. Microbiol. **33**:434–444.
7. **Botsford, J. L., and J. G. Harman.** 1992. Cyclic AMP in prokaryotes. Microbiol. Rev. **56**:100–122.
8. **Bracco, L., D. Kotlarz, A. Kolb, S. Diekmann, and H. Buc.** 1989. Synthetic curved DNA sequences can act as transcriptional activators in *Escherichia coli*. EMBO J. **8**:4289–4296.
9. **Brendel, V., and E. N. Trifonov.** 1984. A computer algorithm testing potential prokaryotic terminators. Nucleic Acids Res. **12**:4411–4427.
10. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: an *in vivo* probe for transcriptional control sequences. Proc. Natl. Acad. Sci. USA **76**:4530–4533.
11. **Ebright, R., P. Cossart, B. Gicquel-Sanzey, and J. R. Beckwith.** 1984. Mutations that alter the DNA sequence specificity of the catabolite gene activator protein of *E. coli*. Nature (London) **311**:232–235.
12. **Ebright, R. H., Y. W. Ebright, and A. Gunasekera.** 1989. Consensus DNA site for the *Escherichia coli* catabolite gene activator protein (CAP): CAP exhibits a 450-fold higher affinity for the consensus DNA site than for the *E. coli lac* DNA site. Nucleic Acids Res. **17**:10295–10305.
13. **Ferrari, E., D. J. Henner, and M. Y. Yang.** 1985. Isolation of an alanine racemase gene from *Bacillus subtilis* and its use for plasmid maintenance in *B. subtilis*. Bio/Technology **3**:1003–1007.
14. **Franklin, F. C. H., and W. A. Venables.** 1976. Biochemical, genetic and regulatory studies of alanine catabolism in *Escherichia coli* K12. Mol. Gen. Genet. **149**:229–237.
15. **Franklin, F. C. H., W. A. Venables, and H. J. W. Wijsman.** 1981. Genetic studies of D-alanine-dehydrogenase-less mutants of *Escherichia coli* K12. Genet. Res. **38**:197–208.
16. **Fukui, K., F. Watanabe, T. Shibata, and Y. Miyake.** 1987. Molecular cloning and sequence analysis of cDNAs encoding porcine kidney D-amino acid oxidase. Biochemistry **26**:3612–3618.
17. **Galactos, N., and C. T. Walsh.** 1987. Specific proteolysis of native alanine racemase from *Salmonella typhimurium*: identification of the cleavage site and characterization of the clipped two-domain protein. Biochemistry **26**:8475–8480.
18. **Galaktos, N. G., E. Daub, D. Botstein, and C. T. Walsh.** 1986. Biosynthetic *alr* alanine racemase from *Salmonella typhimurium*: DNA and protein sequence determination. Biochemistry **25**:3255–3260.
19. **Gartenberg, M., and D. Crothers.** 1988. DNA sequence determinants of CAP-induced bending and protein binding affinity. Nature (London) **333**:824–829.
20. **Gaston, K., A. Bell, A. Kolb, H. Buc, and S. Busby.** 1990. Stringent spacing requirements for transcription activation by CRP. Cell **62**:733–743.
21. **Groisman, E. A., and M. J. Casadaban.** 1986. Mini-Mu bacteriophage with plasmid replicons for in vivo cloning and *lac* gene fusing. J. Bacteriol. **168**:357–364.
22. **Hayashi, H., H. Wada, T. Yoshimura, N. Esaki, and K. Soda.** 1990. Recent topics in pyridoxal 5′-phosphate enzyme studies. Annu. Rev. Biochem. **59**:87–110.
23. **Hennig, J.** 1988. Ph.D. thesis. Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, Warsaw.
24. **Hinton, D.** 1989. Transcript analyses of the *uvsX-40-41* region of bacteriophage T4. Changes in the RNA as infection proceeds. J. Biol. Chem. **264**:14432–14439.
25. **Irani, M., R. Musso, and S. Adhya.** 1989. Cyclic-AMP-dependent switch in initiation of transcription from the two promoters of the *Escherichia coli gal* operon: identification and assay of 5′-triphosphate ends of mRNA by GTP:RNA guanyltransferase. J. Bacteriol. **171**:1623–1630.
26. **Isogai, T., H. Ono, Y. Ishitani, H. Kojo, Y. Ueda, and M. Kohsaka.** 1990. Structure and expression of cDNA for D-amino acid oxidase active against cephalosporin C from *Fusarium solani*. J. Biochem. **108**:1063–1069.
27. **Jones, H., and W. A. Venables.** 1983. Solubilization of D-amino acid dehydrogenase of *Escherichia coli* K12 and its re-binding to envelope preparations. Biochimie **65**:177–183.
28. **Jones, H., and W. A. Venables.** 1983. Effects of solubilization on some properties of the membrane-bound respiratory enzyme D-amino acid dehydrogenase of *Escherichia coli*. FEBS Lett. **15**:189–192.
29. **Kaczorowski, G. J., L. Shaw, M. Fuentes, and C. T. Walsh.** 1975. Coupling of alanine racemase and D-alanine dehydrogenase active transport of amino acids in *Escherichia coli* membrane vesicles. J. Biol. Chem. **250**:2855–2865.
30. **Kaczorowski, G. J., and C. T. Walsh.** 1975. Active transport in *Escherichia coli* B membrane vesicles. Reversible uncoupling by chloropyruvate. J. Biol. Chem. **250**:8931–8937.
31. **Kieny, M. P., R. Lathe, and J. P. Lecocq.** 1983. New versatile cloning and sequencing vectors based on bacteriophage M13. Gene **26**:91–99.
32. **Komeda, Y., and T. Iino.** 1979. Regulation of expression of the flagellin gene (*hag*) in *Escherichia coli* K-12: analysis of *hag-lac* gene fusions. J. Bacteriol. **139**:721–729.
33. **Kyte, J., and R. F. Doolittle.** 1982. A simple method of displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
34. **Lambert, M. P., and F. C. Neuhaus.** 1972. Factors affecting the level of alanine racemase in *Escherichia coli*. J. Bacteriol. **109**: 1156–1161.
35. **Lauferska, U. (Institute of Biochemistry and Biophysics, Warsaw).** 1993. Personal communication.

35a. **Łobocka, M.** Unpublished data.

36. **Mathew, E. (State University of New York—Stony Brook).** 1993. Personal communication.
37. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Momoi, K., K. Fukui, M. Tada, and Y. Miyake.** 1990. Gene expression of D-amino acid oxidase in rabbit kidney. J. Biochem. **108**:406–413.
39. **Momoi, K., K. Fukui, F. Watenabe, and Y. Miyake.** 1988. Molecular cloning and sequence analysis of cDNA encoding human kidney D-amino acid oxidase. FEBS Lett. **238**:180–184.
40. **Mulligan, M. E., D. K. Hawley, R. Entriken, and W. R. McClure.** 1984. *Escherichia coli* promoter sequences predict in vitro RNA polymerase selectivity. Nucleic Acids Res. **12**:789–800.
41. **Olsiewski, P. J., G. J. Kaczorowski, and C. T. Walsh.** 1980. Purification and properties of D-amino acid dehydrogenase, an inducible membrane-bound iron-sulfur flavoenzyme from *Escherichia coli* B. J. Biol. Chem. **255**:4487–4494.
42. **Olsiewski, P. J., G. J. Kaczorowski, C. T. Walsh, and H. R. Kaback.** 1981. Reconstitution of *Escherichia coli* membrane vesicles with D-amino acid dehydrogenase. Biochemistry **20**:6272–6278.
43. **Reitzer, L. J., and B. Magasanik.** 1987. Ammonia assimilation and

the biosynthesis of glutamine, glutamate, aspartate, asparagine, L-alanine, and D-alanine, p. 302–320. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.

44. **Rodier, F., and J. Sallantin.** 1985. Localization of the initiation of translation in messenger RNAs of procaryotes by learning techniques. Biochimie **67**:533–539.
45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
46. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
47. **Schmitz, A.** 1981. Cyclic AMP receptor protein interacts with lactose operator DNA. Nucleic Acids Res. **9**:277–292.
48. **Schultz, G. E.** 1985. Application of computer programs for the structural analysis of proteins, p. 353–369. *In* Modern methods in protein chemistry, vol. 2. Walter de Gruyter & Co., Berlin.
49. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *E. coli* 16S ribosomal RNA complementary to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71**:1342–1346.
50. **Simons, R. W., P. A. Egan, H. T. Chute, and W. D. Nunn.** 1980. Regulation of fatty acid degradation in *Escherichia coli*: isolation and characterization of strains bearing insertion and temperature-sensitive mutants in gene *fadR*. J. Bacteriol. **142**:621–632.
51. **Simpson, R. B.** 1980. Interaction of the cyclic AMP receptor protein with the *lac* promoter. Nucleic Acids Res. **8**:759–766.
52. **Tada, M., K. Fukui, K. Momoi, and Y. Miyake.** 1990. Cloning and expression of a cDNA encoding mouse kidney D-amino acid oxidase. Gene **90**:293–297.
53. **Tanizawa, K., A. Oshima, A. Scheidegger, K. Inagaki, H. Tanaka, and K. Soda.** 1988. Thermostable alanine racemase from *Bacillus stearothermophilus*: DNA and protein sequence determination and secondary structure prediction. Biochemistry **27**:1311–1316.
54. **Thornberry, N. A., H. G. Bull, D. Taub, K. E. Wilson, G. Gimenez-Gallego, A. Rosegay, D. D. Soderman, and A. A. Patchett.** 1991. Mechanism-based inactivation of alanine racemase by 3-halovinylglycines. J. Biol. Chem. **266**:21657–21665.
55. **Walsh, C. T.** 1989. Enzymes in the D-alanine branch of bacterial cell wall peptidoglycan assembly. J. Biol. Chem. **264**:2393–2396.
56. **Wasserman, S. A., E. Daub, P. Grisafi, D. Botstein, and C. T. Walsh.** 1984. Catabolic alanine racemase from *Salmonella typhimurium*: DNA sequence, enzyme purification, and characterization. Biochemistry **23**:5182–5187.
57. **Wasserman, S. A., C. T. Walsh, and D. Botstein.** 1983. Two alanine racemase genes in *Salmonella typhimurium* that differ in structure and function. J. Bacteriol. **153**:1439–1450.
58. **Wild, J., M. Filutowicz, and T. Kłopotowski.** 1978. Utilization of D-amino acids by *dadR* mutants of *Salmonella typhimurium*. Arch. Microbiol. **118**:71–77.
59. **Wild, J., J. Hennig, M. Łobocka, W. Walczak, and T. Kłopotowski.** 1985. Identification of the dadX gene coding for the predominant isozyme of alanine racemase in Escherichia coli K12. Mol. Gen. Genet. **198**:315–322.
60. **Wild, J., and T. Kłopotowski.** 1975. Insensitivity of D-amino acid dehydrogenase synthesis to catabolite repression in *dadR* mutants of *Salmonella typhimurium*. Mol. Gen. Genet. **136**:63–73.
61. **Wild, J., and T. Kłopotowski.** 1981. D-Amino acid dehydrogenase of *Escherichia coli* K12: Positive selection of mutants defective in the enzyme activity and localization of the structural gene. Mol. Gen. Genet. **181**:373–378.
62. **Wild, J., and B. Obrępalska.** 1982. Regulation of expression of the *dadA* gene encoding D-amino acid dehydrogenase in *Escherichia coli*: analysis of *dadA-lac* fusions and direction of *dadA* transcription. Mol. Gen. Genet. **186**:405–410.
63. **Wild, J., B. Zakrzewska, W. Walczak, and T. Kłopotowski.** 1987. Two distinct types of mutations conferring to *Escherichia coli* K12 capability of D-tryptophan utilization. Acta Microbiol. Pol. **36**:17–28.
64. **Young, K. K., and G. Edlin.** Physical and genetic analysis of bacteriophage T4 generalized transduction. Mol. Gen. Genet. **192**:241–246.