JOURNAL OF BACTERIOLOGY, May 1984, p. 535–542
0021-9193/84/050535-08$02.00/0

Vol. 158, No. 2

Cloning, Mapping, and Expression of Genes Involved in the Fatty Acid-Degradative Multienzyme Complex of *Escherichia coli*

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Received 2 December 1983/Accepted 16 February 1984

Two protein subunits (42,000 and 78,000 daltons) encoded by the *fadAB* genes form a multifunctional enzyme complex containing thiolase, 3-hydroxyacyl-coenzyme A dehydrogenase, crotonase, epimerase, and isomerase activities (S. Pawar and H. Schulz, J. Biol. Chem. **256**:3894–3899, 1981). In an attempt to characterize the structural organization and regulatory properties of these genes, a 5.2-kilobase pair fragment containing the *fadAB* genes has been isolated. Plasmids containing this fragment (i) complement mutations in the *fadAB* genes; (ii) overproduce by 10- to 50-fold thiolase, 3-hydroxyacyl-coenzyme A dehydrogenase and crotonase; and (iii) specify a 42,000- and a 78,000-dalton protein. The *fadA* gene, which encodes the 42,000-dalton protein, has been localized within the original clone to a 3.3-kilobase pair fragment. Thiolase activity, which is encoded by the 42,000-dalton protein, was not observed in the absence of the 78,000-dalton protein, suggesting that an intact complex is required for function. Transposon Tn*5* insertional mutagenesis of the cloned *fadAB* genes has demonstrated that both *fadA* and *fadB* are transcribed as a single transcriptional unit with the direction of transcription from *fadA* to *fadB*. The molecular cloning and characterization of the *fadAB* region confirm the original genetic contention that the genes encoding the proteins for the multifunctional complex form an operon.

Growth of *Escherichia coli* on various fatty acids requires the enzymes of the fatty acid-degradative (*fad*) system (Fig. 1). The genes coding for the *fad* enzymes are located at no fewer than four distinct loci on the *E. coli* chromosome and form a regulon (12, 21, 31). The synthesis of at least five *fad* enzymes is coordinately induced when long-chain fatty acids (C_{12} to C_{18}) are present in the growth media (12, 21, 31). At present, all of the available evidence suggests that the product of the *fadR* gene, a diffusible repressor protein, negatively controls the expression of the *fad* regulon (21, 28, 29).

Two of the induced proteins in the *fad* system are associated with a multienzyme complex which has a molecular mass of 260,000 daltons (2, 20, 23, 24). Five *fad* enzyme activities, 3-ketoacyl-coenzyme A thiolase (thiolase), enoyl-coenzyme A hydratase (crotonase), 3-hydroxyacyl-coenzyme A dehydrogenase (HOADH), *cis*-Δ^3-*trans*-Δ^2-enoyl-coenzyme A isomerase (isomerase), and 3-hydroxyacyl-coenzyme A epimerase (epimerase) are associated with this multienzyme complex (2, 23, 24). Schulz and co-workers have purified the complex and found it to have α_2 β_2 subunit structure (α, 78,000 daltons; β, 42,000 daltons) (2, 23, 24). Through biochemical characterization of the multienzyme complex from an *E. coli* B strain, Schulz and co-workers have determined that thiolase activity is associated with the 42,000-dalton subunit, and the remaining four enzyme activities are associated with the larger 78,000-dalton subunit (2, 23, 24).

Overath et al. (21, 22) have suggested that the genes for the enzymes thiolase, HOADH, crotonase, and possibly epimerase and isomerase form an operon. Evidence for the *fadAB* operon was based on the high coordinate induction of thiolase, HOADH, and crotonase as well as on the mapping properties of mutants deficient in (i) all five enzymes (*fad-5* or *fadAB*) (ii) thiolase (*fadA*), and (iii) HOADH (*fadB*) (21, 22). However, genetic evidence that these genes are organized in an operon has not been previously shown.

To investigate the structural organization and the regulation of the *fadAB* genes, we have cloned the *fadAB* genes directly from the *E. coli* chromosome onto a multicopy plasmid. The approximate location and orientation of the *fadA* and *fadB* genes were determined by subcloning and Tn*5* mutagenesis. Our studies support the contention of Overath that the *fadAB* genes are part of an operon, and suggest that the direction of transcription is from *fadA* to *fadB*. The *fadA* and *fadB* gene products have been identified by maxicell analysis, and evidence is presented which suggests that the *fadA* gene product is nonfunctional in the absence of the *fadB* gene product. In strains containing the *fadAB* plasmid, the enzymes thiolase, crotonase, and HOADH are amplified 10- to 50-fold.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* K-12 bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively.

The bacteria were grown in either medium E (18) or LB broth (18) and routinely incubated in a New Brunswick Gyrotory water bath shaker at 37°C. Carbon sources and supplements were sterilized separately and added to culture medium before inoculation. All organic acids were added as neutralized salts. Acetate was provided at a 50 mM final concentration. Where indicated, Trypticase peptone (BBL Microbiology Systems) was provided at 1.0% final concentration. Fatty acids were suspended in 10% Brij 58, neutralized with KOH, sterilized, and added at a final concentration of 5 mM. Bacterial growth was monitored at 540 nm in a Klett-Summerson colorimeter.

Construction of *fadA*, *fadB*, and *fadAB* mutants. *fadAB* mutants were obtained by localized mutagenesis of strain LS5405 (*metE*) by transduction to Met^+ Tc^r (tetracycline resistance) with a P1 *vir* phage stock grown on a random pool of strain K-12 To^r colonies, each individually resistant to

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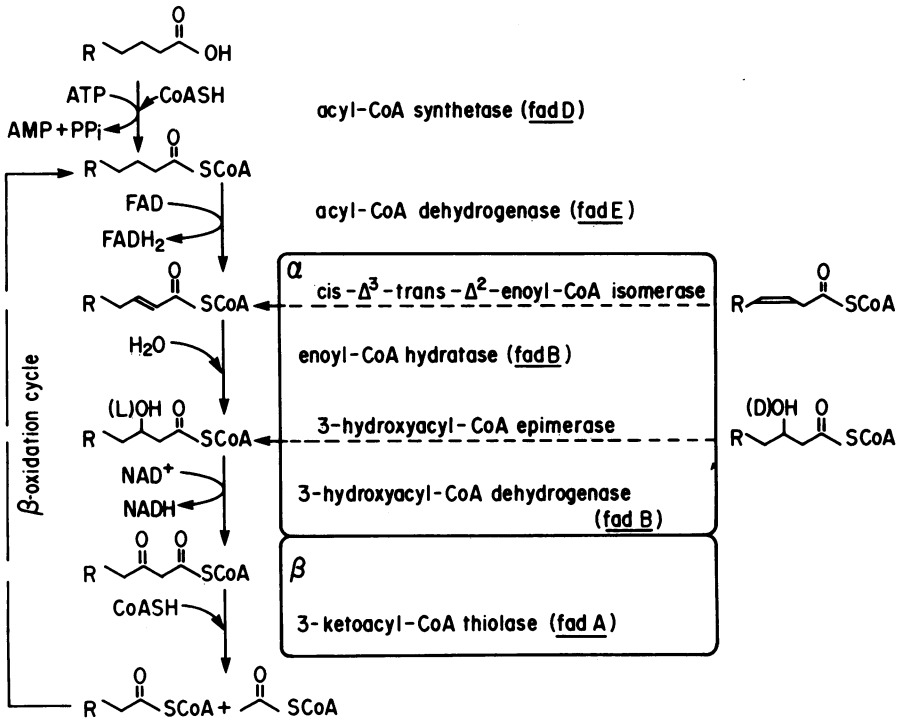

FIG. 1. The cyclic pathway of fatty acid degradation. Principal enzymes of the pathway are listed on the right, along with the respective structural genes of the *fad* regulon. Acetyl-coenzyme A is further metabolized in the tricarboxylic acid cycle.

tetracycline due to the insertion of the transposable element Tn*10* into a different region of the chromosome. Strains containing transposon insertions in the *fadA* or *fadB* gene were isolated by ampicillin enrichment (18) with oleate as the sole carbon source. Surviving cells were plated on media containing acetate as the sole carbon source and were screened for growth on oleate. Isolate LS5786 was unable to grow on oleate and was genetically and biochemically characterized as containing a Tn*10* insertion which eliminated thiolase activity and reduced crotonase and HOADH activities. A P1 *vir* phage stock grown on strain LS5786 was used to transduce LE392 to Tc^r. Both Met^+ Fad^- and Met^- Fad^- transductants were obtained. One Met^- Tc^r Fad^- isolate, designated LS6747, was genetically and biochemically characterized as a *fadAB*::Tn*10* insertion mutant. Strain LS6747 was used to obtain a deletion within the *fadAB* region. A deletion in the *fadAB* locus created by the spontaneous excision of the Tn*10* transposon of LS6747 (*fadAB*::Tn*10*) was obtained by selecting for tetracycline sensitivity as described by Maloy and Nunn (15) and screening the Tc^s isolates for the inability to utilize the fatty acid oleate as a sole carbon source. An Ole^- Tc^s isolate, strain LS6748, which could not revert to Ole^+ or Tc^r, was considered a deletion. Strain LS6748 lacked thiolase and had significantly reduced levels of crotonase and HOADH. A *recA* derivative of strain LS6748, strain LS6749, was the *fadAB* strain we used for cloning and subcloning the *fadAB* genes because it could be transformed 1,000-fold better than a *recA* derivative of the Ymel *fadAB* strain, K1.

Strain LS6577 is a *fadAB* mutant obtained by localized mutagenesis with hydroxylamine hydrochloride as described by Hong and Ames (9). Briefly, a *fad-5* mutant (polar mutation in the putative *fadAB* operon [21]) was transduced to Fad^+ Tc^r with a P1 *vir* phage stock grown on a random pool of strain K-12 Tc^r colonies. A Fad^+ Tc^r isolate, designated LS6524 (*zif*::Tn*10* *fadA*$^+$*B*$^+$), was obtained, and a phage stock was prepared from it. This phage stock was then exposed to 1 M hydroxylamine hydrochloride as described by Hong and Ames (9). The hydroxylamine hydrochloride-mutagenized phage stock was used to transduce strain RS3010 (*fadR*) to Tc^r. This transduction mixture was plated on fatty acid indicator plates (29). Fad^- Tc^r colonies were isolated. One such isolate, designated LS6577, was further purified and characterized as a *fadAB* mutant, and the defect was mapped at 85 min on the recalibrated *E. coli* K-12 linkage map (1).

The *zif*::Tn*10* of strain LS6524 (92% cotransducible with *fadA*$^+$*B*$^+$) was moved into strains containing *fadA30*, *fadB64*, and *fadAB* mutations. P1 *vir* phage stocks of the Tc^r Ole^- derivatives of these strains were used to transduce the host restriction minus strain LE392 to Tc^r Ole^-. Tc^r Ole^- isolates from the individual crosses were isolated and designated LS6595 (*zif*::Tn*10* *fadA*), LS6596 (*zif*::Tn*10* *fadB*), and LS6745 (*zif*::Tn*10* *fadAB*). Fad enzyme activities were measured in these strains to confirm that the correct enzyme defects were present.

When recombination-deficient mutants were required, they were constructed as described by Simons et al. (28).

Isolation and manipulation of DNA. Isolation and purification of plasmid DNA was by the cleared lysate-polyethylene glycol precipitation method of Humphreys et al. (10). Supercoiled plasmid DNA was isolated by dye-buoyant density centrifugation in a cesium chloride gradient containing ethidium bromide (25). Preparation of plasmid DNA from small cultures was performed by the method described by Holmes and Quigley (8). Total chromosomal DNA from bacterial strains was prepared by the method described by Marmur (17).

TABLE 1. Bacterial strains

Strain	Relevant genotype[a] or properties	Reference or source
LE392	*hsdR galK trpR metB lacY*	Cold Spring Harbor
RS3010	*fadR*	Simons et al. (28)
K1	*fad-5*	P. Overath via CGSC[b]
K19	*fadE62*	P. Overath via CGSC
K27	*fadD88*	P. Overath via CGSC
OldA30	*fadA30*	P. Overath via CGSC
OldB64	*fadB64*	P. Overath via CGSC
NK5304	*srlA*::Tn*10 recA*	Nancy Kleckner strain
LS5405	*thi-1 metE68 relA fadR*::Tn*5*	This study
LS5786	*thi-1 metE68 relA fadR*::Tn*5 fadAB*::Tn*10*	This study
LS6491	*metE68 relA fadR*::Tn*5 fadAB*	This study
LS6524	*zif*::Tn*10*[c]	This study
LS6577	*zif*::Tn*10 fadR fadAB*	This study
LS6595	*hsdR galK trpR metB lacY zif*::Tn*10 fadA30*	This study
LS6596	*hsdR galK trpR metB lacY zif*::Tn*10 fadB64*	This study
LS6745	*hsdR galK trpR metB lacY fad-5*	This study
LS6747	*hsdR galK trpR metB lacY fadAB*::Tn*10*	This study
LS6748	*hsdR galK trpR metB lacY fadAB*	This study
LS6749	*hsdR galK trpR metB lacY fadAB recA*	This study
LS7049	*fadL*	Maloy et al. (14)

[a] The nomenclature for genetic symbols follows that of Bachmann and Low (1), and the nomenclature for transpositional insertions follows that of Kleckner et al. (11).

[b] CGSC strains were obtained from B. Bachmann at the *E. coli* Stock Genetic Center, Yale University, New Haven, Conn.

[c] The *zif*::Tn*10* insertion is linked 95% in P1 *vir* transductions to the *fadAB* locus.

Digestion of DNA with restriction endonucleases was generally carried out under the conditions specified by the vendor (Bethesda Research Laboratories). Digestion reactions were terminated by heating from 5 to 10 min at 65°C. Restriction fragments were analyzed by electrophoresis in agarose slab gels prepared in TEA buffer (50 mM Tris, 20 mM sodium acetate, 2 mM disodium EDTA [pH 8.05] [7]).

Recombinant plasmids were constructed in vitro by ligation of endonuclease-generated fragments with T4 DNA ligase (Bethesda Research Laboratories) at 14 to 16°C in 66 mM Tris-hydrochloride (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, and 0.5 mM ATP for 16 to 24 h. DNA concentrations were varied, depending on the desired outcome, in accordance with the consideration of Dugaiczyk et al. (5).

E. coli cells were prepared for transformation with plasmid DNA by the method described by Mandel and Higa (16). Fad^+ transformants were screened on medium E minimal agar plates supplemented with 5 mM oleate and ampicillin (100 μg/ml). Fad^+ transformants appeared in 3 to 4 days at 37°C.

Construction of *pCEM*-derivative plasmids. Derivatives of plasmid pCEM were constructed by the following method. The plasmid vector (pBR322, pACYC177, pK011, or pUC9) and pCEM were individually digested with restriction enzymes, mixed, and then ligated in vitro and T4 DNA ligase. The resulting mixture was used to transform strain LS6749 (*fadAB*). Transformants were obtained by selection for the appropriate antibiotic resistance marker. Hybrid plasmid DNA was isolated, digested with the enzyme previously used, and analyzed as described above.

pCBP was constructed by inserting the *Bam*HI-*Pst*I fragment of pCEM into the ampicillin resistance gene of pACYC177. The *Bam*HI-*Sal*I fragment of pCEM was inserted into the tetracycline resistance gene (*tet*) of pBR322 resulting in plasmid pCBS. The introduction of a *Pst*I-*Sal*I fragment of pCBP into pK011 yielded pK52. Plasmid pKSC contained the *Sal*I-*Cla*I fragment of pCBP in the *tet* gene of pBR322. Plasmid pKPC was constructed by inserting the *Cla*I fragment of pCBP into pBR322.

The *Bgl*II$_1$-*Bgl*II$_4$, *Bgl*II$_1$-*Bgl*II$_3$, *Bgl*II$_1$-*Bgl*II$_2$, *Bgl*II$_2$-*Bgl*II$_3$, and *Bgl*II$_3$-*Bgl*II$_4$ fragments were obtained by *Bgl*II limit digests of pCEM, and subsequent insertions of these fragments into the *Bam*HI site of pUC9 yielded plasmids pK1, pK2, pK3, pK5, and pK6, respectively. The orientation of the *Bgl*II$_1$-*Blg*II$_4$ and *Bgl*II$_1$-*Bgl*II$_3$ fragments were the same relative to the pUC9 sequences, with the B_4 site of pK1 and the B_3 site of pK2 proximal to the *lac* promoter region of pUC9. pK4 was constructed by inserting the *Pst*I-*Bgl*II$_1$ fragment of pCEM into pUC9.

Preparation of cell extracts and enzyme assays. Bacteria were harvested from mid-log-phase cultures (ca. 6.0×10^8 cells per ml), washed three times with ice-cold 100 mM potassium phosphate (pH 7.0), and suspended in 1/40 volume of the same buffer. The cells were disrupted at 4°C in an Aminco French pressure cell at 15,000 lb/in^2. The lysate was centrifuged at 15,000 × *g* for 15 min at 4°C. Protein content of the extract was determined by the Lowry procedure (13) with bovine serum albumin as the standard.

The *fad* enzymes were assayed as previously described (19). Enzyme reactions were monitored in a Beckman recording spectrophotometer at room temperature. All values are the average of at least two determinations.

Isolation of *fadAB*::Tn*5* insertion mutants. Cultures of strain LS6749 (*fadAB*) carrying the recombinant plasmid pK52 (*fadA*$^+$*B*$^+$) were grown to late exponential phase in LB broth and were infected with λ*c*I857 *b*221 *P*am902::Tn*5* at a multiplicity of 10 phage per cell. After incubation for 60 min

TABLE 2. Plasmids

Plasmid	Relevant genotype or properties[a]	Reference or source
pBR322	Ap^r Tc^r	3
pACYC177	Ap^r Km^r	4
pUC9	Ap^r	30
pK011	Ap^r *galK*	6
pCEM	Ap^r Km^r Ole^+	This study
pCBP	Km^r Ole^+	This study
pCBS	Ap^r Ole^+	This study
pK52	Ap^r Ole^+	This study
pKSC	Ap^r *fadAB*	This study
pKPC	Ap^r *fadAB*	This study
pK1	Ap^r *fadA*$^+$*B*$^+$	This study
pK2, pK3, pK4, pK5	Ap^r *fadAB*	This study
pK6	Ap^r *fadAB*	This study
pK52::Tn*5*-1	Ap^r Km^r *fadAB*-1::Tn*5*	This study
pK52::Tn*5*-2	Ap^r Km^r *fadAB*-2::Tn*5*	This study
pK52::Tn*5*-3	Ap^r Km^r *fadAB-3*::Tn*5*	This study

[a] Ap^r, Ampicillin resistant; Tc^r, tetracycline resistant; Km^r, kanamycin resistant.

at room temperature, the infected cells were spread on LB plates containing kanamycin to select for Tn*5* transposition. Kanamycin-resistant (Km^r) colonies (10,000) were pooled and diluted in 30 ml of LB and incubated for 18 h at 37°C. Plasmid DNA was isolated by the small-scale technique and used to transform the kanamycin-sensitive strain LS6749 (*fadAB*) to Km^r. pK52 *fadAB*::Tn*5* insertion mutants were identified by their failure to grow on oleate and by the inability to complement *fadA*, *fadB*, and *fadAB* mutants.

The site of insertion of Tn*5* in pK52 was determined by the distances of restriction sites from the unique and asymmetric *Sal*I and *Sma*I sites within Tn*5* (26).

Maxicells and electrophoresis of proteins. The procedure described previously by Sancar et al. (27) for labeling plasmid-encoded proteins with [^{35}S]methionine was used with the exception that the strain containing the plasmids was LS6749.

Radioactive labeled polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on gradient gels (7.5 to 15% acrylamide [wt/vol] and 0.2 to 0.4% *N*,*N*-methylenebisacrylamide [wt/vol]) with appropriate molecular weight standards in the range of 12,500 to 116,000 to determine molecular weights.

Chemicals. Antibiotics and other chemicals were obtained from Sigma Chemical Co. The various restriction endonucleases and bacteriophage T4 DNA ligase used in this study were obtained from Bethesda Research Laboratories with the exception of *Bgl*II, which was purchased from P-L Biochemicals.

RESULTS

Cloning of *fadAB* from the *E. coli* chromosome. To obtain hybrid plasmids containing the *fadAB* genes, a *Bam*HI genomic digest of *E. coli* K-12 DNA and *Bam*HI-cleaved pACYC177 were mixed, ligated, and then used to transform strain LS6749 (*fadAB*) to ampicillin resistance (Ap^r). Ampicillin-resistant transformants having the Fad^+ phenotype were screened for by their ability to grow on the long-chain unsaturated fatty acid oleate. From a pool of 5,000 Ap^r transformants, 5 Fad^+ transformants were obtained and later found to contain hybrid plasmids with common restriction sites. One Fad^+ Ap^r transformant was found to carry a 13.4-kilobase pair (kb) plasmid. This plasmid, designated pCEM, transformed the original *fad-5* (*fadAB*) mutant of Overath and several independently isolated *fadAB* mutants

TABLE 3. *fad* enzyme activities in *E. coli* strains containing different *fadAB* plasmids

Plasmid[a]	Growth conditions	Strain (genotype)	Genes complemented by plasmid	Sp act[b]			
				Palmitoyl-coenzyme A dehydrogenase	Thiolase	HOADH	Crotonase
None	Uninduced[c]	LE392 (wild type)		0[f]	0.4	26	66
	Induced[d]	LE392 (wild type)		1	5.5	183	333
	Uninduced[c]	LS6749 (*fadAB*)		0	0	0	0
	Induced[d]	LS6749 (*fadAB*)		1	0	17	16
pACYC177	Uninduced[c]	LS6749 (*fadAB*)	None	0	0	0	0
	Induced[d]	LS6749 (*fadAB*)		1.4	0	28	5
pBR322	Induced[e]	LS6745 (*fadAB*)	None	ND[g]	0	0	0
		LS6595 (*fadA*)		ND	0	280	1,680
		LS6596 (*fadB*)		ND	50	0	992
		LE392 (wild type)		ND	14	304	1,613
pCEM	Uninduced[c]	LE392 (wild type)	*fadAB*	0	39	277	2,605
	Induced[d]	LE392 (wild type)		1	197	1,689	7,062
	Uninduced[c]	LS6749 (*fadAB*)		0	33	468	2,187
	Induced[d]	LS6749 (*fadAB*)		1	270	4,223	8,024
	Induced[e]	LS6745 (*fadAB*)		ND	340	1,034	4,232
pCBP	Induced[e]	LS6745 (*fadAB*)	*fadAB*	ND	295	775	4,777
pCBS		LS6745 (*fadAB*)	*fadAB*	ND	926	3,014	9,021
pK52		LS6745 (*fadAB*)	*fadAB*	ND	308	1,745	7,004
pK1		LS6745 (*fadAB*)	*fadA*	ND	0	0	0
		LS6595 (*fadA*)		ND	12	ND	ND
		LS6596 (*fadB*)		ND	ND	0	ND
pK2		LS6745 (*fadAB*)	None	ND	0	0	0
		LS6595 (*fadA*)		ND	10	ND	ND
		LS6546 (*fadB*)		ND	ND	0	ND

[a] See text and Fig. 3 for construction of hybrid plasmids.
[b] Specific activities are expressed in nanomoles/minute per milligram of protein.
[c] Uninduced growth conditions: EB medium (18) supplemented with 1% Trypticase peptone.
[d] Induced growth conditions: EB medium supplemented with 1% Trpticase peptone and 5 mM oleate.
[e] Induced growth conditions: EB medium supplemented with 5 mM oleate and 50 mM potassium acetate.
[f] A measurement of zero implies activity of <0.05.
[f] ND, Not determined.

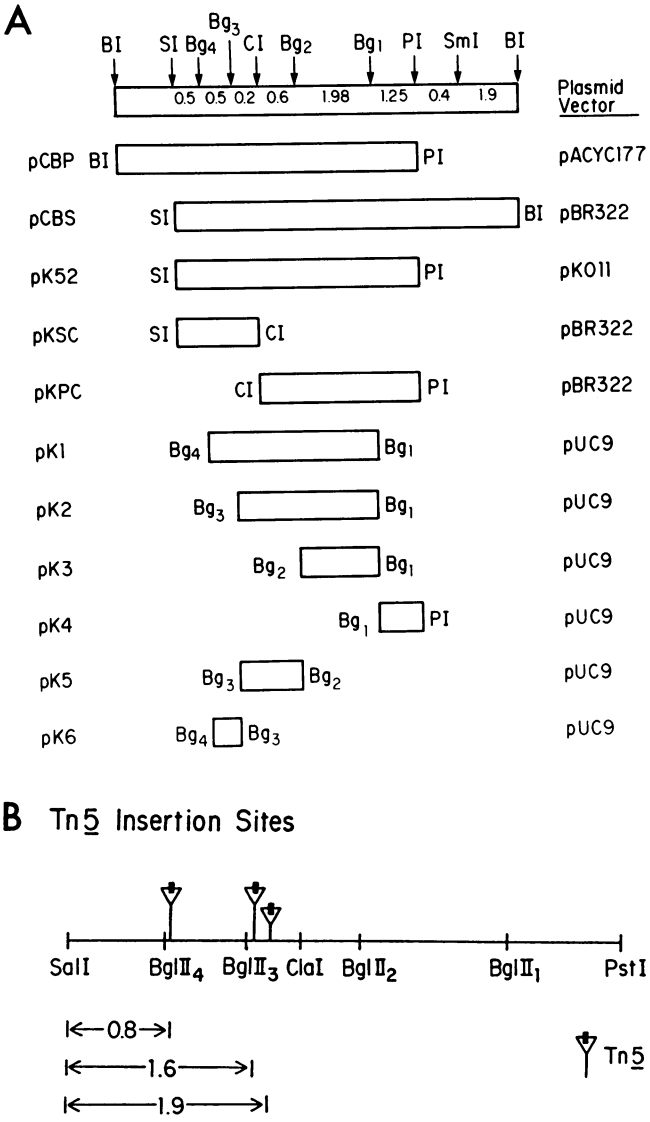

FIG. 2. (A) Physical maps of recombinant plasmids carrying portions of the *fadAB* chromosomal region. At the top is a map of plasmid pCEM whose vector is pACYC177. The orientations of the $BglII_1$-$BglII_4$ and $BglII_1$-$BglII_3$ fragments in pK1 and pK2, respectively, are the same relative to the pUC9 sequences. PI, *Pst*1; PvI, *Pvu*I; BI, *Bam*HI; SI, *Sal*I; Bq, *Bgl*II; CI, *Cla*I; SmI, *Sma*I. (B) Tn*5* insertion map. Tn*5* insertion sites in plasmid pK52 that prevent the expression of the 42,000- and 78,000-dalton proteins. The triangle represents the site of Tn*5* insertion. pK52::Tn*5*-1 (0.8), pK52::Tn*5*-2 (1.6), and pK52::Tn*5*-3 (1.9). (The numbers in parentheses represent the distance between the Tn*5* insertions and the *Sal*I site.)

(LS5786, LS6491, LS6577, LS6745, LS6748, and LS6749) to Ap^r Fad^+ with high efficiency and was presumed to carry the *fadAB* genes. However, pCEM failed to complement *fadE*, *fadL*, and *fadD* mutations, indicating that the plasmid did not have a pleiotropic effect on other genes of the *fad* regulon.

To confirm the presence of the genes encoding the enzymes thiolase, crotonase, and HOADH, the activities of these three enzymes were measured in a *fadAB* mutant (LS6749) containing plasmid pCEM. As shown in Table 3, the three enzyme activities were restored whenever pCEM was present in a *fadAB* mutant. Strains containing pCEM (LE392 and LS6749) overproduced the three activities (Table 3) by 10 to 50-fold when compared with the parental strain LE392. Because *fadAB* strains containing pCEM were able to grow on or degrade the long-chain unsaturated fatty acid oleate, we expected that the plasmid should also encode the enzymes epimerase and isomerase, two additional enzymes required for the complete oxidation of monosaturated and polyunsaturated fatty acids. H. Schulz (personal communication) found that epimerase and isomerase activities were also present and amplified in a *fadAB* strain (LS6749) containing pCEM, confirming that the plasmid not only encoded thiolase, HOADH, and crotonase but also epimerase and isomerase. Furthermore, these enzyme activities were inducible (Table 3), suggesting that the *fadAB* genes present on the plasmid are regulated by the same mechanism(s) which controls the expression of the *fad* regulon (21, 28, 29). The activity of the *fad* enzyme acyl-coenzyme A dehydrogenase, whose gene, *fadE*, is not linked to the *fadAB* genes, was not affected by the presence of pCEM (Table 3). The activity of the acyl-coenzyme A synthetase, the product of the unlinked *fadD* gene, was also not affected by the presence of pCEM (data not shown).

Restriction map of pCEM. As a first step toward establishing the gene organization of pCEM, a restriction map was constructed. The single *Hin*dIII, *Sma*I, and *Pst*I cleavage sites on pACYC177 were used as reference points for the location of other restriction sites. The plasmid pCEM was 13.4 kb of which 9.4 kb is *E. coli* chromosomal DNA inserted into the *Bam*HI site of pACYC177. The cloned region contained single unique cleavage sites for *Pst*I, *Sal*I, *Cla*I, and *Sma*I; four *Bgl*II sites; and no sites for *Ava*I, *Hin*dIII, *Eco*RI, *SSt*I, and *SSt*II (Fig. 2A).

Subcloning the *fadAB* genes. To develop a physical map of the *fadAB* region, various restriction endonuclease-generated fragments were subcloned into pACYC177, pBR322, pK011, and pUC9. The genotypes of the derivative plasmids were determined by complementation of the *fadA*, *fadB*, and *fadAB* defects. Among the pCEM-derivative plasmids, only pCBP, pCBS, and pK52 complemented the *fadAB* defect in strain LS6745 (Table 4). Plasmid pK52 contained the smallest subcloned restriction fragment (*Pst*I-*Sal*I) which comple-

TABLE 4. Genetic complementation of various plasmids with strains carrying the *fadA30*, *fadB64*, or Δ*fadAB* mutation[a]

Plasmid	Insert size (kb)	Vector	Complementation with strain:		
			LS6595 (*fadA30*)	LS6596 (*fadB64*)	LS6745 (*fadAB*)
pCEM	9.4	pACYC177	+	+	+
pCBS	7.1	pBR322	+	+	+
pCBP	7.0	pACYC177	+	+	+
pK52	5.2	pK011	+	+	+
pKSC	1.2	pBR322	−	−	−
pKPC	3.8	pBR322	−	−	−
pK1	3.3	pUC9	+	−	−
pK2	2.7	pUC9	−	−	−
pK3	1.98	pUC9	−	−	−
pK4	1.25	pUC9	−	−	−
pK5	0.8	pUC9	−	−	−
pK6	0.5	pUC9	−	−	−

[a] Mutant strains were transformed with plasmid DNA, and transformants were selected at 37°C on minimal media supplemented with 5 mM oleate and ampicillin (100 μg/ml). Complementation was determined after 3 to 4 days of incubation.

mented the *fadAB* defect. Further evidence attesting to the presence of the *fadAB* genes on the *Pst*I-*Sal*I fragment was obtained from enzymological studies which showed elevated levels of thiolase, crotonase, and HOADH activites in an *fadAB* strain containing either plasmids pCBP, pCBS, or pK52 (Table 3).

Subclones containing restriction fragments smaller than the 5.2-kb *Pst*I-*Sal*I fragment (pKPC, pKSC, pK1 to pK6) failed to complement the *fadAB* and *fadB* mutations (Table 4). However, plasmid pK1 complemented the *fadA* defect but not the *fadB* and *fadAB* defects, suggesting that the *fadA* gene resides in the 3.3-kb $BglII_1$-$BglII_4$ fragment. Enzymological studies showed that pK1 restores wild-type levels of thiolase activity to a *fadA* but not to a *fadAB* strain (Table 3). When the 5.2-kb *Pst*I-*Sal*I fragment was cleaved at the internal *Cla*I site, resulting subclones pKSC and pKPC failed to complement the *fadA* defect (Table 4) or restore thiolase activity to a *fadA* mutant (data not shown). These findings suggest that the *Cla*I site resides within the *fadA* gene. It should also be noted that comparable results were obtained with these plasmids in *recA*$^-$ derivatives of these *fadA*, *fadB*, and *fadAB* strains (data not shown).

Since the above studies indicate that plasmid pK1 carried the *fadA* gene and plasmid pK52 encoded for both *fadA* and *fadB*, the results showing that pK1 conferred thiolase activity to *fadA* but not *fadAB* strains (Table 3) suggest that the *fadA* gene product may not be functional in the absence of the *fadB* gene product(s).

Surprisingly, plasmid pK2 failed to complement the *fadA* defect but did restore thiolase activity to a *fadA* strain (Tables 3 and 4). These results suggest that the 0.5-kb $BglII_3$-$BglII_4$ fragment contains an essential portion of the *fadA* gene required for complementation.

Identification of the *fadAB* gene products. The maxicell procedure (27) was used to detect plasmid-encoded proteins from pCEM-derivative plasmids and to correlate these products with specific functions. Two polypeptides with molecular masses of 42,000 and 78,000 daltons were consistently expressed from the plasmids pCBP and pK52 in strain LS6749 (Fig. 3A). A 42,000-dalton protein was expressed from the *fadA*$^+$ plasmid pK1 (Fig. 3B). This is consistent with earlier findings of Schulz and co-workers (2, 23, 24) which indicated that thiolase activity is associated with a 42,000-dalton protein. The 50,000- to 60,000-dalton protein bands which were consistently observed in irradiated strains with and without plasmids (Fig. 3A and B) appear to be host-encoded proteins (Fig. 3B, lane 1).

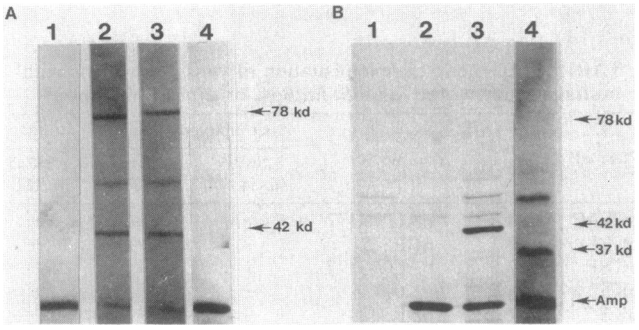

FIG. 3. Autoradiogram of [^{35}S]methionine-labeled plasmid-encoded proteins in maxicells. Maxicells were prepared and plasmid-encoded proteins were analyzed on 7.5 to 15% sodium dodecyl sulfate gradient polyacrylamide gels. (A) Individual lanes represent labeled samples as follows: 1, pACYC177; 2, pCBP; 3, pK52; and 4, pK52::Tn*5*-1. (B) Individual lanes represent labeled samples as follows: *1*, strain LS6749 containing no plasmids; 2, pUC9; 3, pK1; and 4, pK2. When the proteins of pK52::Tn*5*-2 and *pK52::Tn5*-3 were analyzed, comparable results to those with pK52::Tn*5*-1 were obtained (data not shown). The strain used for maxicell analysis was LS6749. The protein products are expressed in kilodaltons.

The plasmid pK2 expressed a 37,000-dalton protein (Fig. 3B, lane 4). We suspect that this protein may be a truncated 42,000-dalton protein, and this may explain why pK2 failed to complement the *fadA* defect (Table 4). To date, we have not isolated a restriction fragment which only complements the *fadB* defect.

Insertional inactivation of the *fadAB* genes. To further define the structural organization of the genes encoding the 42,000- and 78,000-dalton proteins, plasmid pK52 was mutagenized with the transposon Tn*5*. Mutagenesis with the Km^r transposon Tn*5* can destroy gene function by (i) insertional inactivation of a gene(s) and (ii) prevention of the expression of a gene(s) distal to the site of the insertion (26). Derivatives of pK52 carrying random Tn*5* insertions were screened for their ability to complement *fadA*, *fadB*, and *fadAB* mutants. Three Tn*5* insertions that inactivated the *fadAB* were clustered in a 1.3-kb region situated between $BglII_2$-$BglII_4$ (Fig. 2B). The position and orientation of the Tn*5* insertion were determined by restriction endonuclease digestion with *Sal*I, *Bgl*II, and *Cla*I.

fadA, *fadB*, and *fadAB* strains carrying Tn*5* insertions in the *fadAB* genes of plasmid pK52 do not grow on oleate and also lack thiolase, crotonase, and HOADH enzyme activities (data not shown). Furthermore, the 42,000-dalton and 78,000-dalton proteins were not expressed from these plasmids (Fig. 3A, lane 4; pK52::Tn*5*-1). The fact that all *fadAB*::Tn*5* insertion mutants failed to grow on oleate and did not produce the two proteins and the fact that the original site of insertion was within the *fadA* gene suggest that both proteins are produced from the same transciptional unit. The polar inactivation of the Tn*5* insertion mutants indicates that the direction of transcription of the unit is from *fadA* to *fadB*.

DISCUSSION

We have cloned the *fadAB* genes onto a multicopy plasmid as a first attempt to understand more precisely the structural and regulatory properties of the putative *fadAB* operon initially described by Overath et al., (21, 22). By a combination of subcloning, complementation analysis, enzymology, and gene product labeling, a 5.2-kb *Pst*I-*Sal*I fragment was shown to contain the *fadAB* genes. Plasmids bearing these genes were found to encode the genetic information for the 42,000- and 78,000-dalton protein subunits of a multienzyme complex which has thiolase, HOADH, and crotonase activities. Since plasmids bearing the 5.2-kb *Pst*I-*Sal*I fragment also restore isomerase and epimerase to *fadAB* mutants (Schulz, personal communication) our studies strongly support the contention of Schulz et al. (2, 23, 24) that the multienzyme complex, consisting of two 42,000- and two 78,000-dalton subunits, has five *fad* enzyme activities.

The *fadA* coding region has been localized to a 3.3-kb $BglII_1$-$BglII_4$ fragment. This conclusion is supported by the fact that this fragment expresses a 42,000-dalton protein and restores thiolase activity to a *fadA* mutant (Fig. 3B and Table 3). These findings are consistent with the studies of Schulz and co-workers (2, 23, 24), which suggest that the 42,000-dalton protein subunit has the thiolase activity. We have not obtained a fragment which encodes solely the *fadB* gene.

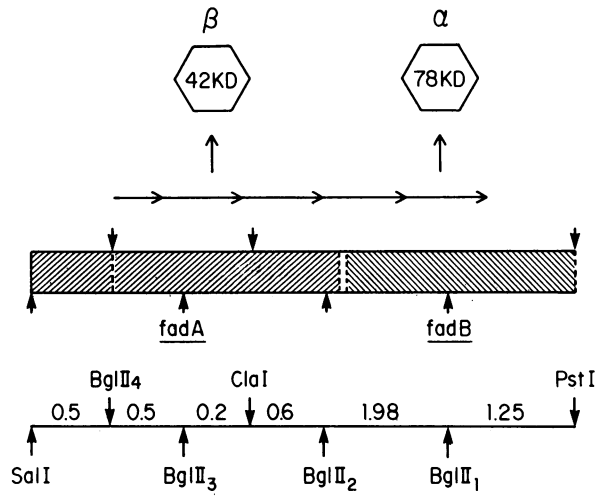

FIG. 4. Structural organization and direction of transcription of the *fadAB* region. The relative location (shaded areas) of the *fadA* and *fadB* genes and the direction of transcription are indicated. The endpoints of these genes are not precisely defined and are indicated by a broken line. The protein products (expressed in kilodaltons [KD]) specified by the genes are given at the top of the figure. Restriction sites *Pst*I, *Bgl*II, *Cla*I, and *Sal*I are indicated. The size of the restriction fragments is also given. This figure is not drawn to scale.

However, since the *Pst*I-*Sal*I fragment of plasmid pK52 contains both *fadA* and *fadB* and since *fadA* lies within the 3.3-kb *Bgl*II$_1$-*Bgl*II$_4$ fragment, the *fadB* gene must lie within the overlapping *Pst*I-*Bgl*II$_2$ fragment since no other fragment can provide continuous coding sequences with the capacity (2.1 kb) to encode the *fadB* gene product. Further evidence supporting this suggestion comes from our studies which show that Tn*5* insertions in *fadA* prevent the expression of *fadB*.

All fragments smaller than the 3.3-kb *Bgl*II$_1$-*Bgl*II$_4$ fragment failed to complement either *fadA*, *fadB*, or *fadAB* mutations. However plasmid pK2, containing a 2.7-kb pair *Bgl*II$_1$-*Bgl*II$_3$ fragment, did restore thiolase activity to a *fadA* mutant (Table 3). Plasmid pK2 was constructed by inserting the 2.7-kb pair *Bgl*II$_1$-*Bgl*II$_3$ fragment of pCEM into *Bam*HI-cut pUC9. The vector pUC9 contains the transcriptional and translational start sequences from the *lacZ* gene upstream of the *Eco*RI site (30). Genes inserted in correct translational frame may be expressed as a fusion product under the control of the *lac* promoter (Halfman et al., unpublished data). We suspect that a portion of the *fadA* gene in pK2 is inserted in a correct translational frame yielding a fusion product of 37,000 daltons consisting of the *lacZ* amino terminus fused to a truncated version of the *fadA* gene product. Nevertheless, these results indicate that a portion of the 0.5-kb *Bgl*II$_3$-*Bgl*II$_4$ fragment contains coding sequences which are required for complementation of the *fadA* defect.

Our results support the suggestions of Overath et al. (21, 22) and Shultz et al. (2, 23, 24) that the *fadAB* genes form an operon. In this study, we present evidence which suggests that the *fadAB* genes are transcribed as a single transcriptional unit and the direction of transcription is from *fadA* to *fadB*. Tn*5* insertions in the *fadA* gene resulted in the loss of both the 42,000 and 78,000 subunits (Fig. 3A), accounting for the loss of activities for thiolase, HOADH, and crotonase (data not shown). A Tn*5* insertion in *fadA* is thought to prevent the expression of *fadB* because of the strong polar effects characteristic of transposon insertion mutations (26). The fact that a single Tn*5* insertion in *fadA* prevents the expression of the *fadB* gene product (78,000-dalton protein) suggests that *fadA* and *fadB* are transcribed as a single transcriptional unit. Therefore, we believe that the relative location and orientation of the *fadA* and *fadB* genes on the 5.2-kb insert are as illustrated in Fig. 4. If *fadA* and *fadB* form an operon and if *fadB* is distal to the promoter as our studies suggest, our inability to date to obtain restriction fragments which complement only the *fadB* defect may be explained by the separation of the *fadB* gene from its promoter.

It is interesting that *fadAB* strains containing the plasmid pK1 synthesize the 42,000-dalton β subunit (Fig. 3B) but do not have thiolase activity (Table 3). One explanation for this finding is that the *fadA* gene product, the β subunit, may not be functional in the absence of the *fadB* gene product, the 78,000-dalton protein (α subunit). Some support for this belief comes from studies which show that *fadA* mutants carrying plasmids (pCBP or pK52) express both the 78,000- and 42,000-dalton proteins and have amplified levels of thiolase activity, whereas those containing the *fadA*$^+$ plasmid (pK1) express the 42,000-dalton protein and have wild-type levels of thiolase activity (Table 3). It appears that, in the latter case, the *fadA* mutant, containing plasmid (pK1) and high levels of the 42,000-dalton protein, may be restricted from expressing amplified levels of thiolase activity because it has only haploid levels of the *fadB* gene product. Additional support for this contention comes from studies showing that wild-type strains harboring the plasmids pK1 or pK2 or both have comparable levels of thiolase activity to wild-type strains which either contain no plasmid or the plasmid vector pUC9 (Nunn, unpublished data).

ACKNOWLEDGMENTS

We thank C. L. Ginsburgh, Karen Oishi, and Hung Fan for helpful discussion and advice. We thank Barbara Bachmann for generous gifts of bacterial strains. We gratefully acknowledge the technical assistance of Kelleen Giffin and D. D. O'Brien for the construction of *fadAB* strains. We thank Cynthia Verdecia for typing the manuscript.

The work was supported by Public Health Service grant GM 22466-08 from the National Institutes of Health. W.D.N. is an Established Investigator of the American Heart Association, and S.K.S. was supported by postdoctoral fellowship GM 09221-02 from the National Institute of General Medical Science.

LITERATURE CITED

1. **Bachmann, B. J., and K. B. Low.** 1980. Linkage map of *Escherichia coli* K-12, Edition 6. Microbiol. Rev. **44**:1–56.
2. **Binstock, J. F., A. Pramanik, and H. Schulz.** 1977. Isolation of a multienzyme complex of fatty acid oxidation from *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **74**:492–495.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlack, H. L. Heynecker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
4. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
5. **Dugaiczy, K. A., H. Winfield, and H. M. Goodman.** 1975. Ligation of EcoRI endonuclease generated DNA fragments into linear and circular structures. J. Mol. Biol. **96**:171–184.
6. **Hauser, C. A., and G. W. Hatfield.** 1983. Nucleotide sequence of the *ilvB* multivalent attenuator region of *Escherichia coli* K-12. Nuc. Acid Res. **11**:127–139.

7. **Helling, R. B., H. M. Goodman, and H. W. Boyer.** 1974. Analysis of Endonuclease R · *Eco*RI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. J. Virol. **14**:1235–1244.
8. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**:93–95.
9. **Hong, J. S., and B. N. Ames.** 1971. Localized mutagenesis of any specific small region of the bacterial chromosomes. Proc. Natl. Acad. Sci. U.S.A. **68**:3158–3162.
10. **Humphreys, G. O., G. A. Willshaw, and E. S. Anderson.** 1975. A simple method for the preparation of large quantities of pure plasmid DNA. Biochim. Biophys. Acta **383**:457–463.
11. **Kleckner, N., J. Roth, and D. Botstein.** 1977. Genetic engineering *in vivo* using translocatable drug-resistance elements. J. Mol. Biol. **116**:125–129.
12. **Klein, K., R. Steinberg, B. Fiethen, and P. Overath.** 1971. Fatty acid degradation in *Escherichia coli*: an inducible system for the uptake of fatty acids and further characterization of *old* mutants. Eur. J. Biochem. **19**:442–450.
13. **Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. **Maloy, S. R., C. L. Ginsburgh, R. W. Simons, and W. D. Nunn.** 1981. Transport of long chain fatty acids by *Escherichia coli* K-12. J. Biol. Chem. **256**:3735–3742.
15. **Maloy, S. R., and W. D. Nunn.** 1981. Selection for the loss of tetracycline resistance by *Escherichia coli*. J. Bacteriol. **145**:1110–1112.
16. **Mandel, M., and A. Higa.** 1970. Calcium dependent bacteriophage DNA infection. J. Mol. Biol. **53**:154–162.
17. **Marmur, J.** 1961. A procedure for the isolation of DNA from microorganisms. J. Mol. Biol. **3**:208–218.
18. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Nunn, W. D., R. W. Simons, P. A. Eagan, and S. R. Maloy.** 1979. Kinetics of the utilization of medium and long chain fatty acids by a mutant of *Escherichia coli* defective in the *fadL* gene. J. Biol. Chem. **254**:9130–9134.
20. **O'Brien, W. J., and F. E. Frerman.** 1977. Evidence for a complex of three beta-oxidation enzymes in *Escherichia coli*; induction and localization. J. Bacteriol. **132**:532–540.
21. **Overath, P., G. Paul, and H. U. Schairer.** 1969. Fatty acid degradation in *Escherichia coli*. An inducible acyl-CoA synthetase, the mapping of *old* mutations, and the isolation of regulatory mutants. Eur. J. Biochem. **7**:559–574.
22. **Overath, P., and E. Raufuss.** 1967. The induction of the enzymes of fatty acid degradation in *Escherichia coli*. Biochem. biophys. Res. Commun. **29**:28–33.
23. **Pawar, S., and H. Schulz.** 1981. The structure of the multienzyme complex of fatty acid oxidation from *Escherichia coli*. J. Biol. Chem. **256**:3894–3899.
24. **Pramanik, A., S. Pawar, E. Antonian, and H. Schulz.** 1979. Five different enzymatic activities are associated with the multienzyme complex of fatty acid oxidation from *Escherichia coli*. J. Bacteriol. **137**:469–473.
25. **Radloff, R., W. Bauer, and J. Vinograd.** 1967. A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. **57**:1514–1521.
26. **Rothstein, S. J., R. A. Jorgensen, K. Postle, and W. S. Reznikoff.** 1980. The inverted repeats of TN5 are functionally different. Cell **19**:795–805.
27. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-coded proteins. J. Bacteriol. **137**:692–693.
28. **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 mutations in gene *fadR*. J. Bacteriol. **142**:621–632.
29. **Simons, R. W., K.T. Hughes, W. D. Nunn.** 1980. Regulation of fatty acid degradation in *Escherichia coli*: dominance studies with strains merodiploid in gene *fadR*. J. Bacteriol. **143**:726–730.
30. **Vieifa, J., and J. Messing.** 1982. The pUC plasmids, and M13mp7-derived systems for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
31. **Weeks, G., M. Shapiro, R. O. Burns, and S. J. Wakil.** 1969. Control of fatty acid metabolism. 1. Induction of the enzymes of fatty acid oxidation in *Escherichia coli*. Eur. J. Biochem. **97**:827–836.