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Regulation of Fatty Acid Degradation in *Escherichia coli*: Isolation and Characterization of Strains Bearing Insertion and Temperature-Sensitive Mutations in gene *fadR*

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Transposon Tn*10* was used to mutagenize the *fadR* gene in *Escherichia coli*. Mutants bearing *fadR*::Tn*10* insertion mutations were found to (i) utilize the noninducing fatty acid decanoate as sole carbon source, (ii) β-oxidize fatty acids at constitutive rates, and (iii) contain constitutive levels of the five key β-oxidative enzymes. These characteristics were identical to those observed in spontaneous *fadR* mutants. The constitutive phenotype presented by the *fadR*::Tn*10* mutants was shown to be genetically linked to the associated transposon-encoded drug resistance. These results suggest that the *fadR* gene product exerts negative control over the fatty acid degradative regulon. The *fadR* gene of *E. coli* has been mapped through the use of transposon-mediated *fadR* insertion mutations. The *fadR* locus is at 25.5 min on the revised map and cotransduces with *purB*, *hemA*, and *trp*. Three-factor conjugational and transductional crosses indicate that the order of loci in this region of the chromosome is *purB-fadR-hemA-trp*. Spontaneous *fadR* mutants were found to map at the same location. Strains that exhibit alterations in the control of the *fad* regulon in response to changes in temperature were also isolated and characterized. These *fadR*(Ts) mutants were constitutive for the *fad* enzymes at elevated temperatures and inducible for these activities at low temperatures. The *fadR*(Ts) mutations also map at the *fadR* locus. These results strongly suggest that the *fadR* gene product is a repressor protein.

Wild-type *Escherichia coli* K-12 is able to grow on long-chain (12 or more carbons) but not on short- (C_4 to C_5) or medium- (C_6 to C_{11}) chain fatty acids as a sole carbon source (21, 22, 26). Growth in media containing long-chain fatty acids induces the coordinate synthesis of at least five key enzymes involved in the β-oxidation of fatty acids (21, 22, 26). Mutants unable to grow on fatty acids of any chain length have been obtained and shown to harbor lesions in structural genes for the β-oxidative enzymes (21, 22) as well as for genes involved in fatty acid activation (11, 15) and transport (11, 19, 20). These fatty acid degradation (*fad*) lesions map at no fewer than four separate locations on the *E. coli* chromosome (15, 19, 21). Strains harboring a lesion in one of the structural genes, *fadD*, lack fatty acyl-coenzyme A synthetase activity and cannot be induced for the other β-oxidative enzymes (15). These results led Klein et al. (15) to propose that long-chain acyl-coenzyme As serve to induce the fatty acid degradative (*fad*) system in *E. coli*.

Medium-chain fatty acids can serve as substrates for the β-oxidative enzymes, but cannot themselves induce the *fad* system (21, 22, 26). However, mutants able to utilize medium- as well as long-chain fatty acids as the sole carbon source can be readily selected by plating the wild type onto media containing the medium-chain fatty acid decanoate (C_{10}) (21, 26). These mutants are found to possess constitutive levels of the five key β-oxidative enzymes (21, 26). Overath et al. (21) proposed that these strains harbored lesions in a gene coding for some regulatory element, possibly a repressor, and they termed this gene *fadR*.

The most common phenotype resulting from mutations in a gene coding for a repressor is that of constitutivity, or derepression. The noninducible phenotype is rarer. In contrast, mutations in a gene coding for an activator protein most frequently result in non-inducibility, the constitutive mutant being quite rare (6). Although the exact nature of the *fadR* gene product is unknown, spontaneous decanoate-utilizing mutants (*fadR*) are present at reasonably high frequencies (ca. 2×10^{-6}) in a variety of strains (27; Simons and Nunn, unpublished data), suggesting that the *fadR* gene might code for a negative controlling element. If this is so, then insertion of a translocatable element into the

fadR gene would result in the severe mutagenesis of the *fadR* locus, the constitutive synthesis of the *fad* enzymes, and the ability of the cells to grow on decanoate (Dec$^+$). On the other hand, if the *fadR* gene codes for a positive controlling element, the inserted transposon would result in non-inducibility of the *fad* enzymes and the inability of the cells to grow on fatty acids of any chain length.

This paper reports the successful selection of mutants that harbor an insertion of Tn*10* (transposon carrying tetracycline resistance, Tc^r [13]) in the *fadR* gene. A genetic and biochemical characterization of these *fadR*::Tn*10* mutants shows them to be similar in all respects to spontaneous *fadR* strains, with the exception of the presence of the transposon-mediated drug resistance. These studies show that insertion mutations in the *fadR* gene result in the constitutive synthesis of the *fad* enzymes, rather than non-inducibility, implying strongly that the *fadR* gene product is indeed a repressor molecule. By the use of these *fadR*::Tn*10* mutants, we have confirmed the observation of Vanderwinkel et al. (24) that the *fadR* gene maps in the 25-min region of the *E. coli* chromosome, and we have also established a more precise location for this gene. We also report the isolation and characterization of mutants that are constitutive for the *fad* enzymes at elevated temperatures and inducible for these activities at low temperatures. These *fadR*(Ts) mutations map at the *fadR* locus. We have therefore adopted as a working model Overath's earlier suggestion that the *fadR* gene product is a repressor operating to negatively control transcription of the *fad* structural genes.

MATERIALS AND METHODS

Phage and bacterial strains. λ NK370 (obtained from D. Botstein), which carries a Tn*10* insertion in the *cI* gene, the *b221* deletion, and an opal mutation in the *O* gene, was used in all translocations of Tn*10* in this work (12). The transducing phage P1 *vir* was obtained from J. Cronan, Jr. All bacterial strains were derivatives of *E. coli* K-12 and are listed in Table 1. NK5336 was the host strain for λ NK370. Strain RS3010 was a spontaneous *fadR* mutant of K-12, selected on decanoate (Dec$^+$). RS3032, RS3044, and RS3046 are *fadR*::Tn*10* mutants, all selected from the insertion mutagenesis of JK268, as described below. Transducing phage P1 *vir*, grown on RS3032, was used to transduce K-12 to tetracycline resistance (Tc^r), and a Dec$^+$ Tc^r isolate, RS3040, was purified for further study. RS3039 was obtained by transducing SASX41B with phage P1 *vir* grown on RS3032, followed by selection for tetracycline resistance. A *purB fadR13*::Tn*10 hemA* derivative (RS3039) was then isolated. RS3042 was a spontaneous *fadR* derivative of JK268, selected on decanoate. The *purB fadR hemA* strain, RS3059, was obtained in the following manner. Strain H680 was infected with phage P1 *vir* grown on SASX41B followed by selection for *trp*$^+$, and a *hemA* transductant was isolated. This transductant was in turn plated onto decanoate, and the spontaneous *fadR* strain RS3059 was obtained. The *fadR*(Ts) strain, RS3066, was obtained from the following enrichment. Approximately 10^8 cells of strain K-12 were spread onto each of 10 plates containing potassium decanoate (5 mM) as the sole carbon source. Incubation at 42°C for 48 h gave rise to ca. 200 colonies per plate. These colonies were then scraped from the plates, suspended in 10 ml of minimal medium M9, and blended in a Vortex mixer. Cells from this pool were then inoculated at a density of ca. 5×10^7 cells per ml into medium M9 supplemented with potassium decanoate (5 mM), and the cultures were incubated at 25°C, with aeration, through two cell doublings. At this point, ampicillin was added at a final concentration of 80 μg/ml, and incubation was allowed to continue for ca. 8 h, at which time lysis was complete. The lysed culture was centrifuged at $6,000 \times g$ for 20 min, the pellet was suspended in 1.0 ml of M9, and 0.1 ml was used to inoculate 10 ml of medium M9 supplemented with decanoate. This culture was then grown out at 42°C, with aeration, for 30 to 48 h. This ampicillin enrichment was repeated serially, two additional times, and cells from the final out-growth were plated for single colonies onto dextrose. These colonies were then replica-plated for determination of their ability to grow on potassium decanoate or potassium oleate at 25 and 42°C. Of 180 colonies so screened, 9 were found to grow well on oleate at either temperature but to grow well on decanoate only at 42°C. One of these strains was purified and designated RS3066.

Strain RS3079 was obtained in the following way. Strain HfrH was infected with λ NK370 selecting for Tc^r as previously described (12). Approximately 10^6 Tc^r colonies were pooled and used to grow the transducing phage P1 *vir*. This P1 *vir*-HfrH::Tn*10* pool lysate was then used to transduce JK268, followed by the simultaneous selection for both Tc^r and *purB*$^+$. One of these transductants, RS3079, was found by genetic analysis to harbor a Tn*10* insertion in the region of the main chromosome between *purB* and *fadR*. This insertion was termed *bee-101*::Tn*10* for its proximity to the 25-min position on the *E. coli* genetic map (5). The first two letters (*be*) designate the 25-min region of the *E. coli* genetic map, and the third letter (*e*) designates the *E. coli* chromosome.

To construct RS3097, strain RS3066 was first transduced with P1 *vir* grown on RS3079, and then, after selection for Tc^r, a *fadR*(Ts) (i.e., able to grow on decanoate at 42°C but not at 25°C) transductant was isolated and termed RS3066 *bee-101*::Tn*10*. Strain K-12 was then transduced with P1 *vir* grown on RS3066 *bee-101*::Tn*10*, selecting for Tc^r, and the Tc^r *fadR*(Ts) strain (RS3097) was purified. In effect, the *bee-101*::Tn*10* insertion was used to "move" the *fadR*(Ts) lesion into the K-12 background without placing selective pressure on the *fadR* gene itself. The construction of strains in this fashion, by the use of Tn*10* insertions near a gene of interest, has been discussed by Kleckner et al. (14). The isogenic strains RS3098 and RS3099 were constructed as follows. Strain RS3010 was transduced with P1 *vir* grown on strain RS3079 followed by

TABLE 1. *Bacterial strains employed*

Strain	Sex	Relevant genotype[a]	Source
H680	F⁻	*purB51 trp-45 his-68 tyrA2 thi-1 rpsL125*	P. G. de Haan strain via CGSC[b]
JC1552	F⁻	*leu-6 trp-31 his-1 argG6 metB*	A. J. Clark strain via CGSC
JK268	F⁻	*purB58 trpE61 trpA62*	J. Kuhn strain (8) via CGSC
K12	F⁺	Prototrophic	J. Lederberg strain via CGSC
KL208	Hfr	Prototrophic	K. B. Low strain (17) via CGSC
LA2-89	F⁻	*fabD1 thi-1 gltA*	Harder et al. (9) via J. Cronan
LA12	F⁺	*ptsG21 thi-1*	B. Magasanik strain (16) via CGSC
MA1008	Hfr	*pyrC46*	W. Maas strain (2) via CGSC
NK5336	F⁻	*su⁺uga*	N. Kleckner strain
RS3010	F⁺	*fadR1*	This work
RS3032	F⁻	*purB58 fadR13*::Tn*10 trpE61 trpA62*	This work
RS3039	Hfr	*purB58 fadR13*::Tn*10 hemA42 metB1*	This work
RS3040	F⁺	*fadR13*::Tn*10*	This work
RS3042	F⁻	*purB58 fadR11 trpE61 trpA62*	This work
RS3044	F⁻	*purB58 fadR14*::Tn*10 trpE61 trpA62*	This work
RS3046	F⁻	*purB58 fadR15*::Tn*10 trpE61 trpA62*	This work
RS3059	F⁻	*purB51 fadR12 hemA41 his-68 tyrA2 thi-1 rpsL125*	This work
RS3066	F⁺	*fadR41*	Temperature sensitive, this work
RS3079	F⁻	*trpE61 trpA62 bee-101*::Tn*10*[c]	This work
RS3097	F⁺	*fadR41 bee-101*::Tn*10*	This work
RS3098	F⁺	*bee-101*::Tn*10*	This work
RS3099	F⁺	*fadR1 bee-101*::Tn*10*	This work
SASX41B	Hfr	*hemA41 metB1*	A. Sasarman strain via CGSC

[a] Only relevant and auxotrophic markers are listed; see references for complete genotype. Allele numbers of *fadR* mutations and Tn*10* insertions are ours; all others are those of the source.

[b] CGSC, Coli Genetic Stock Center, Yale University, New Haven, Conn.

[c] Isolation and nomenclature of this Tn*10* insertion are described in the text.

selection for Tc^r. Two transductants were isolated: one was Tc^r, *fadR*$^+$ and was termed RS3098, and the other was Tc^r, *fadR1* and was termed RS3099. All other strains have been described.

Media and growth conditions. Minimal medium E (25) and rich broth (18) were used for routine growth of bacteria. For some of our temperature-sensitive mutant studies, minimal medium M9 (18) was employed. Lambda broth contained 10 g of tryptone (Difco) and 2.5 g of NaCl per liter. Lambda-YM medium was lambda broth supplemented with 0.2% maltose and 0.01% yeast extract (Difco). SM medium was 0.02 M Tris-hydrochloride buffer (pH 7.5), containing 0.1 M NaCl and 0.01 M $MgSO_4$. LB medium contained 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter. Lambda broth plates and lambda broth top agar contained 1.0% and 0.65% agar (Difco), respectively. All other plates contained 1.5% agar (Difco). Carbon sources and supplements were sterilized separately and added to media before inoculation. All fatty acids were suspended in 10% Brij 58, saponified by neutralization with KOH, and filter-sterilized. Potassium acetate and D-glucose were used at 80 mM and 30 mM, respectively, and fatty acids were provided at 5 mM in the presence of Brij 58 (5 mg/ml). Amino acids were added at 100 μg/ml, and adenine and δ-aminolevulinic acid were provided at 40 μg/ml. Tetracycline was usually added at 20 μg/ml, and kanamycin was used at 30 μg/ml. Bacterial cultures were routinely incubated in a New Brunswick gyratory water bath-shaker at 37°C, and cell growth was monitored at 540 nm in a Klett-Summerson colorimeter. Growth studies were performed essentially as described (20).

Genetic methods. Preparation of phage stocks and transductions were as described (19). The conjugation of KL208 and RS3059 was performed, essentially as described by Miller (18), for 45 min at 37°C, and the cells were plated without interruption with streptomycin (100 μg/ml) for counter-selection. Selection for *hemA*$^+$ was performed with acetate (80 mM) as carbon source. The *ptsG* marker was scored on solid indicator plates containing 5-bromo-4-chloro-indolyl-β-D-galactoside (20 μg/ml), as described by Tyler et al. (23).

Scoring of Dec$^+$ and Dec$^-$ phenotypes. In some strain backgrounds more than others, we found it difficult to distinguish the Dec$^+$ and Dec$^-$ phenotypes clearly. In all cases where such a distinction was not clear, one or more of the following methods were employed. First, we found that growth of replica-plated colonies at 37°C for 12 to 18 h, followed by continued growth at 25°C, was often sufficient for distinction. Second, assessment of colony color on the indicator plates described below usually gave excellent distinction. If doubt remained after these procedures, the strains were purified and examined by testing for β-oxidation in vivo.

Indicator plates. To differentiate between *fadR*$^+$ and *fadR* colonies, a modification of the general solid indicator plate reported by Bochner and Savageau (3) was used. This plate contained potassium octanoate (5 mM), Brij 58 (5 mg/ml), 2,3,5-triphenyl tetrazolium chloride (0.0025%), proteose peptone (0.2%), and agar (Difco; 1.5%) in minimal medium E. Colonies able to

metabolize octanoate (*fadR*) turned red on these plates; those unable to do so (*fadR*$^+$) remained white.

Translocation of Tn*10*. The translocation of Tn*10* from λ NK370 to the *E. coli* chromosome was accomplished essentially according to the method of Kleckner et al. (12). The simultaneous selection for tetracycline-resistant, decanoate-utilizing (Tc^r, Dec$^+$) colonies was accomplished by plating onto medium E containing 5 mM decanoate, Brij 58 (5 mg/ml), 2.5 mM sodium pyrophosphate, and tetracycline at 20 μg/ml after infection with NK370.

Enrichment for tetracycline-sensitive revertants. Strain RS3040 was pregrown in 20 ml of medium E supplemented with glucose (30 mM) to ca. 1.5×10^8 cells per ml. Tetracycline was then added to a concentration of 4 μg/ml, and growth was allowed to continue for 60 min. The culture was then split; one 10-ml portion received 0.4 ml of ampicillin (0.5 mg/ml, resulting in 20 μg/ml final concentration), and the remaining 10-ml control received 0.4 ml of medium E. Growth in the control was followed through two doublings, at which time lysis was complete in the enrichment culture. The enriched culture was centrifuged at 10,000 × *g* for 10 min at 4°C, washed once with 0.5 volume of medium E, and suspended in 1.0 ml of medium E, and then 0.1 ml was subcultured into 10 ml of medium E supplemented with glucose (30 mM) for outgrowth. The entire enrichment procedure was then serially repeated twice. The final enriched, glucose-grown culture was then plated for single colonies on rich broth which were in turn replicated onto oleate, decanoate, and tetracycline plates. A total of 890 colonies were tested in this way; 160 (18%) were found to be tetracycline sensitive (Tc^s). Of these 160, all were Dec$^+$.

Assay of β-oxidation. Fatty acid oxidation was assayed in vivo by determining the amount of $^{14}CO_2$ formed from 1-^{14}C-labeled fatty acids as described by Weeks et al. (26), with the exception that chloramphenicol was present (100 μg/ml) during the assay procedure.

Assay of β-oxidative enzyme activities. The five key β-oxidative enzyme activities were assayed from whole-cell extracts as described previously (20). Cell fractionation and protein determinations were made as described previously (20).

Materials. All fatty acids were used without further purification. All unlabeled fatty acids except hexanoate were purchased from Sigma Chemical Co., St. Louis, Mo. Hexanoic acid was purchased from Calbiochem, La Jolla, Calif. All 1-^{14}C-labeled fatty acids were purchased from Dhom Products, Hollywood, Calif., except *cis*-9-[1-^{14}C]octadecenoic acid (oleic acid), which was a product of New England Nuclear Corp., Boston, Mass. Ampicillin, tetracycline hydrochloride, kanamycin sulfate, 2,3,5-triphenyl tetrazolium chloride, 5-bromo-4-chloro-indolyl-β-D-galactoside, and Brij 58 were purchased from Sigma. Chloramphenicol was a product of Calbiochem. Trypticase peptone was purchased from BBL Microbiology Systems, Cockeysville, Md. All other reagents employed were of reagent grade.

RESULTS

Mutagenesis of the *fadR* gene with the translocatable drug resistance element Tn*10*. The translocation of Tn*10* to the *E. coli* chromosome was accomplished by selecting for drug resistance after infection of cells with λ NK370 under conditions where the bacteriophage genome can neither replicate nor lysogenize, according to the method of Kleckner et al. (12). After infection of strain JK268, when selection was made only for tetracycline resistance (Tc^r), the frequency of drug-resistant colonies per infecting phage genome (1.7×10^{-5}) was similar to published frequencies of Tn*10* translocation in *E. coli* (7, 12). As expected, when individual Tc^r "transductants" were examined further, approximately 1% were found to be auxotrophic (requirements not identified). However, no Dec$^+$ cells were found among 700 Tc^r transductants screened. In a separate experiment, selection was made simultaneously for both Tc^r and the ability to grow on decanoate as the sole carbon source. The rationale was to select directly for insertion of Tn*10* into the *fadR* gene, under the assumption that the *fadR* gene product was a repressor. In this case the frequency of transductants was much lower (1.9×10^{-9} per infecting phage genome) than when drug resistance alone was selected, as would be expected for the insertion mutagenesis of a specific gene. A ratio of these two frequencies shows that one would expect only about 1.1×10^{-4} Tc^r transductants to be Dec$^+$. This result is in agreement with the published prediction for the frequency of Tn*10* insertion into a single gene (14). Three Tc^r Dec$^+$ transductants (RS3032, RS3044, and RS3046) were isolated for further studies.

If the Tc^r Dec$^+$ strains described above did indeed arise from the insertion of Tn*10* into the *fadR* gene, then the drug resistance and Dec$^+$ phenotypes of these strains should be genetically linked, and this linkage should be absolute. Accordingly, bacteriophage P1 *vir* were grown on the putative *fadR* insertion mutants, RS3032, RS3044, and RS3046, and then were used to donate drug resistance in transductions with strain K-12. When 180 drug-resistant transductants from each cross were then replica-plated onto decanoate, all were found to be Dec$^+$, demonstrating linkage between these two phenotypes. Moreover, in a variety of genetic crosses involving the Tc^r marker that originated in strain RS3032, the Tc^r and Dec$^+$ phenotypes were not found to segregate in some 3,000 tested recombinants (data summarized in Table 4). These results suggest that the lesion in these strains is *fadR*::Tn*10*.

Biochemical characterization of *fadR*::Tn*10* mutants. Spontaneous *fadR* lesions have been shown to extend the range of fatty acids that can serve as a sole carbon source to include

the medium-chain-length homologs (C_7 to C_{11}) (20, 21, 26). Growth studies were performed with strains RS3010 (*fadR1*), RS3040 (*fadR13*::Tn*10*), and K-12 (*fadR*$^+$) to determine whether the *fadR*::Tn*10* mutation had a similar effect. The results showed that *fadR1* and *fadR13*::Tn*10* strains exhibited very similar growth constants on the fatty acids C_6 through C_{18}, in contrast to the parent strain, K-12, which grew only on C_{12} or longer fatty acids (data not shown). K-12, RS3010, and RS3040 did not differ in their ability to grow on a variety of other carbon sources, such as acetate, glycerol, succinate, D-glucose, or various rich broths (data not shown). Moreover, when strains RS3010 and RS3040 were grown in minimal media containing 80 mM acetate (with Brij 58 [5 mg/ml]) as a carbon source, and then washed and subcultured into media containing one of the fatty acid homologs C_6 to C_{18} (5 mM, with Brij 58 [5 mg/ml]) as the carbon source, no significant lag in growth was observed. In contrast, strain K-12, although able to grow on homologs C_{12} to C_{18}, did so only after a lag of 1.8 to 2.5 h when shifted from acetate (data not shown).

Spontaneous *fadR* mutants have also been shown to possess constitutive levels of the β-oxidative enzymes, allowing the cells to oxidize various fatty acids at high rates, whether grown under inducing or noninducing conditions. In contrast, *fadR*$^+$ cells are able to carry out substantial oxidation of fatty acids only after growth under inducing conditions (21, 22, 26). To compare the ability of *fadR*$^+$, *fadR*, and *fadR*::Tn*10* strains to oxidize fatty acids under various conditions, several in vivo β-oxidation experiments were carried out. In the first experiment, strains K-12, RS3010, and RS3040 were grown under noninducing conditions (acetate as the carbon source) and then examined for their ability to oxidize several medium-chain fatty acids. Strains RS3010 (*fadR1*) and RS3040 (*fadR13*::Tn*10*) oxidized octanoate, nonanoate, decanoate, and undecanoate at comparable rates, which were from five to eight times higher than rates exhibited by the parent, K-12 (*fadR*$^+$). These results (data not shown) suggested that RS3010 and RS3040 possess comparable levels of β-oxidative enzymes, and that these levels are sufficient for the oxidation of medium-chain fatty acids. In a second experiment (Table 2), K-12, RS3010, and RS3040 were grown under noninducing (acetate), inducing (acetate + oleate), or catabolite-repressing (D-glucose) conditions and then tested for the ability to oxidize either decanoate or oleate. The results of this experiment (Table 2) showed that the rates of decanoate or oleate oxidation by strains RS3010 and RS3040 were nearly identical, whether the cells were grown under inducing or noninducing conditions. Strain K-12, on the other hand, oxidized these fatty acids only after induction with oleate. Fatty acid oxidation is catabolite repressed by glucose in all three strains (Table 2). The results of these in vivo β-oxidation studies clearly indicate that the *fadR*::Tn*10* lesion, like the spontaneous *fadR* mutations, endows the cell with constitutive levels of the *fad* enzymes and that these enzymes remain catabolite repressible.

TABLE 2. *Oxidation of decanoate and oleate by K-12, RS3010, and RS3040*

Strain	Pregrowth carbon source[a]	Rate of release of CO_2 (nmol/min per mg) from:	
		[1-^{14}C]decanoate	[1-^{14}C]-oleate
K-12	D-Glucose	0.15	0.11
	Acetate	1.76	1.07
	Acetate + oleate	10.88	10.95
RS3010	D-Glucose	1.86	1.29
	Acetate	9.45	9.93
	Acetate + oleate	10.7	9.97
RS3040	D-Glucose	1.70	1.24
	Acetate	10.86	10.55
	Acetate + oleate	12.07	11.83

[a] Cells were grown in medium M9, supplemented with carbon sources at the following concentrations: D-glucose, 30 mM; potassium acetate, 80 mM; potassium oleate, 5 mM. All cultures contained Brij 58 (5 mg/ml).

To test the prediction of the β-oxidation studies above, whole-cell extracts from K-12, RS3010, and RS3040, grown under various conditions, were assayed for the activities of the five key β-oxidative enzymes. The results (Table 3) show that, whether grown under inducing or noninducing conditions, extracts from RS3010 and RS3040 have constitutive levels of the β-oxidative enzyme activities, comparable to levels in extracts from induced K-12 cells. In addition, catabolite repression of these activities by growth in D-glucose was observed in all three strains. These results confirm that the *fadR13*::Tn*10* lesion in RS3040 derepresses the *fad* system to the same extent as does the spontaneous *fadR1* mutation in RS3010. It is this constitutivity of the *fad* enzymes in strains RS3010 and RS3040 that allows growth on the noninducing medium-chain substrates.

Mapping of the *fadR* locus. Vanderwinkel et al. (24) have mentioned preliminary unpublished data that the *fadR* locus cotransduces about 35% with the *purB* marker in *E. coli*. Accordingly, strain RS3032 (*purB fadR13*::Tn*10*) was transduced with P1 *vir* grown on K-

TABLE 3. *Specific activities of β-oxidative enzymes from extracts of K-12, RS3010, and RS3040*[a]

Strain	Carbon source[b]	Oleoyl-CoA[c] synthetase	Palmitoyl-CoA dehydrogenase	Crotonase	β-Hydroxybutyryl-CoA dehydrogenase	Acetoacetyl-CoA thiolase
K-12	D-Glucose	0.09	0.24	<5.0	<5.0	1.5
	Acetate	0.33	0.31	88	36	3.6
	Acetate + oleate	1.98	3.8	688	539	40
RS3010	D-Glucose	0.04	1.4	158	89	8.6
	Acetate	1.69	3.96	942	942	44
	Acetate + oleate	1.87	4.61	647	1,128	64
RS3040	D-Glucose	0.10	1.1	110	161	5.7
	Acetate	1.72	4.76	915	1,058	29
	Acetate + oleate	1.87	5.74	675	997	46

[a] Enzyme activities are expressed in nanomoles per minute per milligram of protein.
[b] Cells were pregrown in medium M9 supplemented with carbon sources at the following concentrations: D-glucose, 30 mM; potassium acetate, 80 mM; potassium oleate, 5 mM. All cultures contained Brij 58 (5 mg/ml).
[c] CoA, Coenzyme A.

12 (*purB*$^+$ Dec$^-$), followed by selection for *purB*$^+$ recombinants. When examined by replica-plating, about 45% of the *purB*$^+$ colonies were found to be tetracycline sensitive (Tc^s), and in addition were unable to grow on decanoate as the sole carbon source (Dec$^-$). A similar frequency of cotransduction between *purB*$^+$ and *fadR1* was observed in crosses between RS3010 and JK268. These results confirmed those of Vanderwinkel et al. (24) and supported our contention that the insertion mutation in RS3032 was indeed *fadR*::Tn*10*. A more precise location of the *fadR* locus was determined by examining cotransduction between *fadR* and various other loci in the 23- to 27-min region (Table 4). The *fadR* locus was found to be cotransduced at a fairly high frequency with *hemA*, at a modest frequency with *ptsG* and *fabD*, and very rarely or not at all with *trp* and *pyrC*. It should be noted that in crosses where the donor carried the *fadR*::Tn*10* marker, cotransduction with flanking markers was reduced. This would be expected as a result of the increased separation of markers by the inserted Tn*10* element. The order of *purB*, *fadR*, and *hemA* was determined in crosses involving all three loci. The results of three different three-factor crosses are presented in Table 5. In each three-factor cross, the relative frequencies of the four possible recombinant classes should be inversely related to the minimum number of crossovers required for their formation. The cotransductional data in Table 4 and the three-factor cross results in Table 5 strongly favor the gene order *purB-fadR-hemA*. We therefore suggest the placement of the *fadR* locus at ca. 25.5 min on the revised *E. coli* linkage map (18), as shown in Fig. 1.

Reversion of tetracycline resistance in RS3040. Insertion of the Tn*10* transposon not only inactivates the gene in which insertion has occurred, but also exerts a strong polar effect on the expression of operator-distal genes in the same operon (4). Although it was felt that the Dec$^+$ and Tc^r phenotypes of RS3040 and related strains were the result of direct insertion of Tn*10* into the *fadR* gene, a polarity effect on the *fadR*$^+$ gene was an equally plausible explanation. The Tc^r phenotype of a number of Tn*10* insertions has been shown to revert to tetracycline sensitivity at low frequencies (ca. 10^{-4}) as a result of the excision of part or all of the transposon from the point of insertion (4). Such excision events occur predominantly in an imprecise fashion, often but not always causing deletion of a part of the inserted gene. These imprecise excision events thereby result in sensitivity to tetracycline, but not in restoration of gene activity. On the other hand, any polarity that is present is frequently relieved as a consequence of these imprecise excision events (4, 7). We therefore enriched RS3040 for Tc^s revertants in the presence of ampicillin (20 μg/ml) and low levels of tetracycline (4 μg/ml). When 160 Tc^s colonies obtained in this way were examined, all were found to be Dec$^+$. Several such Tc^s revertants were tested and found to oxidize oleate at constitutive levels (data not shown). We believe that these Dec$^+$ Tc^s revertants are the result of the imprecise excision of part or all of the Tn*10* transposon from the *fadR* gene, resulting in *fadR* deletions or other host chromosomal rearrangements. More importantly, the results tend to rule out polarity as an explanation for the Dec$^+$ phenotype in RS3040.

Isolation and genetic characterization of thermally inducible *fadR* mutants. The genetic and biochemical data presented above suggested that *fadR* may be the structural gene for

TABLE 4. *P1 cotransduction frequencies*[a]

Selected donor marker	Inheritance of unselected donor marker (% cotransduction)						
	pyrC	*ptsG*	*fabD*	*purB*	*fadR*[b]	*hemA*	*trp*
pyrC$^+$				4.0	<0.6		
purB$^+$	2.1	22			45 (17)	4.1	<0.4
fadR13::Tn*10*	<0.4	3.2	3.5	27		35	<0.4
hemA$^+$				6.4	37 (15)		
trp$^+$				<0.4	0.8 (<0.4)		

[a] All markers are from strains listed in Table 1. At least 180 colonies were scored in each transduction.

[b] The mutation used in most of these measurements was *fadR1*. The results in parentheses are from crosses with *fadR13*::Tn*10*. The *fadR* mutations in strains RS3044 and RS3046, as well as 10 independently obtained spontaneous *fadR* mutants, were also examined, and all were found to be 43 to 54% cotransducible with *purB*.

TABLE 5. *Three-factor crosses between purB, fadR, and hemA*

Cross[a]	Donor / Recipient				Selected marker (no. scored)	Resulting characteristics			Relative frequency (% of total)
						purB	*fadR*	*hemA*	
1	K-12	+	+	+	*purB*$^+$ (356)	+	+	+	6.2
						+	+	−	40.2
	RS3039	*purB58*	*fadR13*::Tn*10*	*hemA41*		+	−	+	3.4
						+	−	−	50.2
					hemA$^+$ (89)	+	+	+	16.8
						−	+	+	58.4
						+	−	+	9
						−	−	+	15.7
2	SASX41B	+	+	*hemA41*	*purB*$^+$ (231)	+	+	+	43.7
						+	+	−	2.6
	RS3042	*purB58*	*fadR11*	+		+	−	+	53.7
						+	−	−	0
3[b]	KL208	+	+	+	*purB*$^+$ (237)	+	+	+	68.0
						+	+	−	25.3
	RS3059	*purB51*	*fadR12*	*hemA41*		+	−	+	2.5
						+	−	−	4.2
					hemA$^+$ (267)	+	+	+	59.1
						−	+	+	14.3
						+	−	+	6.4
						−	−	+	20.2

[a] Crosses 1 and 2 were mediated by P1 transduction, and cross 3 was mediated by conjugation. The proximity and direction of transfer of KL208 (*hemA fadR purB*) may account for the rather low frequency of the *purB*$^+$ *fadR hemA* class in cross 3.

[b] The frequencies of recombination between markers in this cross were *purB-fadR*, 6.7%; *purB-hemA*, ca. 32%; and *fadR-hemA*, 27%. Given a correlation of 1 min = 20 recombination units (10), these values are equivalent to 0.34, 1.6, and 1.35 min, respectively.

a regulatory protein, probably a repressor, which exerts negative control over the *fad* regulon. This suggestion received further support from the isolation and characterization of *fadR* mutants in which the *fad* enzymes are thermally inducible. These mutants were obtained from a pool of Dec$^+$ mutants enriched for the ability to utilize decanoate at 42°C, but not at 25°C (see Materials and Methods). One of these mutants, RS3066, exhibited a clear temperature-dependent phenotype and was characterized further. Genetic studies showed that the lesion in RS3066 responsible for this temperature-dependent Dec$^+$ phenotype cotransduced with *purB* (45%), and that its order with respect to other markers in the region was identical to that of *fadR* (data not shown). The isogenic strains RS3097, RS3098, and RS3099 were constructed

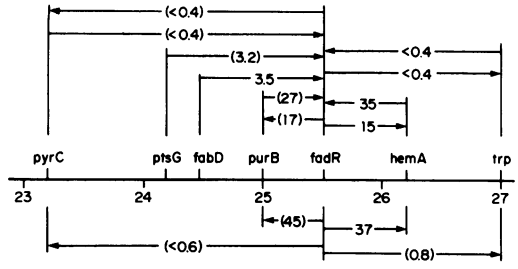

FIG. 1. *Location of the fadR gene on the genetic linkage map of E. coli. A scaled portion of the map, from 23 to 27 min, is illustrated here. It was adapted from the revised E. coli linkage map of Bachmann et al. (1). Values shown above the map are results of transductional crosses where the donor carried the fadR13::Tn10 allele. Values below the map are results of crosses where fadR⁺ or fadR1 alleles were donated. Values in parentheses are nonweighted averages of two or more crosses. Arrowheads point to the selected marker. All values are percent cotransduction and are based on data from Table 4.*

(see Materials and Methods) and used in the biochemical studies reported below.

Growth behavior of a thermally inducible strain. The growth behavior of these isogenic strains was examined under various conditions. The putative *fadR*(Ts) strain RS3097 was unable to grow appreciably on medium-chain fatty acids (C_6 to C_{11}) at 25°C, but at 37°C it grew at rates comparable to the *fadR* strain RS3099. The *fadR*⁺ strain RS3098 was unable to grow on medium-chain fatty acids at either temperature. All three strains grew at comparable rates on acetate or oleate. These results (data not shown) suggested that the ability of RS3097 to grow on medium-chain fatty acids is temperature dependent, a characteristic not observed in the other strains.

To determine the effects of a change in incubation temperature upon the growth of these strains on decanoate, samples from cultures of RS3097, RS3098, and RS3099 were withdrawn during logarithmic growth at 25°C, and growth was subsequently followed at 37°C. Similarly, samples from cultures growing at 37°C were withdrawn and followed at 25°C. The results are shown in Fig. 2. The constitutive strain RS3099 continued to grow well on decanoate when the incubation temperature was changed, although growth was somewhat slower at 25°C. The *fadR*⁺ strain RS3098 did not begin growth on decanoate when shifted to 25°C, or to 37°C, as expected. However, when a sample of the putative *fadR*(Ts) strain RS3097, growing very slowly at 25°C, was shifted to 37°C, rapid growth began, but only after a lag of about 3 h. If the lesion in RS3097 manifests itself as a themolabile repressor of *fad* gene expression, then this 3-h

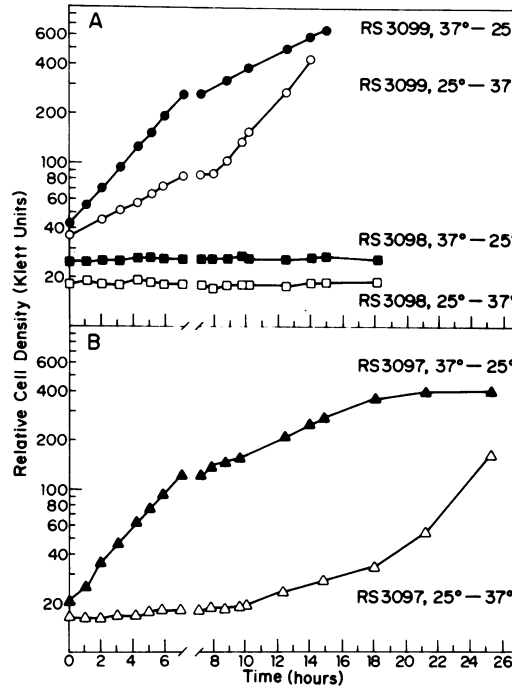

FIG. 2. *Effect of temperature change during the growth of RS3097, RS3098, and RS3099 on decanoate. Strains were grown at the indicated temperatures in medium M9 supplemented with potassium decanoate (5 mM) and Brij 58 (5 mg/ml) as described in the text. Growth was followed at the initial temperature for ca. 7 h, at which time (denoted by break in curve) the culture was divided into equal portions. One portion was returned as a control for growth at the initial temperature (controls not shown), and the other was allowed to grow at a different temperature. In some instances, cultures were diluted into appropriately prewarmed M9-Brij-decanoate media to avoid entry into stationary phase. Such dilutions were taken into consideration in determination of the relative cell densities. All control cultures were found to grow at the same rate as observed before temperature shift. (A) (●) RS3099 (fadR⁻) grown initially at 37°C, then shifted to 25°C; (○) RS3099 grown initially at 25°C, then shifted to 37°C; (■) RS3098 (fadR⁺) grown at 37°C, then at 25°C; (□) RS3098 grown at 25°C, then at 37°C. (B) (▲) RS3097 [fadR(Ts)] grown initially at 37°C, then shifted to 25°C; (△) RS3097 grown at 25°C, then at 37°C.*

lag before rapid growth at 37°C would represent the period of time required for the synthesis of the *fad* enzymes after thermal induction. A lag of similar duration was observed during the induction of growth of RS3099 on oleate (data not shown). In contrast, when cultures of RS3097, growing well on decanoate at 37°C, were shifted to 25°C, growth continued at a somewhat lower rate, but slowed considerably after several generations (Fig. 2). These latter results with strain

RS3097 can be interpreted to mean that, upon shifting to 25°C, repression of the *fad* genes is restored, *fad* enzyme synthesis ceases, and continued growth occurs for several generations until dilution or degradation of these enzymatic activities has occurred.

In vivo β-oxidation of fatty acids by a thermally inducible strain. The ability of strain RS3097 to β-oxidize oleate in vivo was also found to be temperature dependent (Table 6). This strain oxidized oleate at increased rates when grown in the presence of oleate, or at elevated temperatures in the presence or absence of oleate, but not when grown on acetate or Trypticase peptone at 25°C. Therefore, this strain is inducible (by oleate) at 25°C and partially constitutive at 37°C. The partial constitutivity at 37°C is further inducible by oleate. In contrast, the *fadR*$^+$ strain RS3098 oxidized oleate at a high rate only when grown in the presence of oleate. Strain RS3099 was constitutive for the oxidation of oleate at both temperatures. All three strains are catabolite repressed by D-glucose. It is interesting that a moderate temperature-dependent increase in the rate of oleate oxidation by strain RS3099 was observed when acetate was present during growth. This effect was also seen in RS3098 grown on acetate + oleate. However, neither strain showed the effect when grown on oleate or Trypticase peptone alone. Moreover, acetate appears to repress the constitutivity in RS3097 or RS3099 (Table 6).

TABLE 6. *Oxidation of oleate by RS3097, RS3098, and RS3099*

Strain	Carbon source[a]	Rate of release of CO_2[b] from cells pregrown at:	
		25°C	37°C
RS3097 [*fadR*(Ts)]	Acetate	1.6	6.5
	Acetate + oleate	8.6	10.8
	Oleate	17.9	17.7
	Trypticase peptone	2.2	15.1
	D-Glucose	—[c]	0.8
RS3098 (*fadR*$^+$)	Acetate	0.6	0.6
	Acetate + oleate	6.2	12.8
	Oleate	17.7	21.7
	Trypticase peptone	1.7	1.8
	D-Glucose	—	0.3
RS3099 (*fadR*)	Acetate	5.4	10.6
	Acetate + oleate	7.1	14.0
	Oleate	19.5	20.1
	Trypticase peptone	19.2	19.4
	D-Glucose	—	2.5

[a] Cells were pregrown at the indicated temperature in medium M9 supplemented with carbon sources at the following concentrations: potassium acetate, 80 mM; potassium oleate, 5 mM; D-glucose, 30 mM. All cultures contained Brij 58 (5 mg/ml).

[b] Rates expressed in nanomoles per minute per milligram of protein, determined at 37°C.

[c] —, Not determined.

The temperature-dependent phenotype of strain RS3097 might also be explained by proposing that some form of activation of the β-oxidative enzymes occurs at elevated temperatures, although the growth studies presented above argue against this. In all three strains, when cells harvested from cultures grown at 25 or 37°C were examined for their rate of oleate oxidation at 37°C, these rates were consistently about twofold higher than the rates exhibited by the same cells assayed at 25°C (data not shown). This most likely reflects the general temperature dependence of biochemical reactions, and is in agreement with the faster rate of growth of these strains on oleate at 37°C (Fig. 2). More importantly, these results showed that cells obtained from cultures of RS3097 grown at 25°C (or 37°C) did not exhibit an increase in the rate of oleate oxidation beyond this twofold change.

Thermal induction of the β-oxidative enzymes. The results of the experiments above are consistent with our proposal that the lesion in RS3097 (originating in RS3066) renders the *fadR* gene product temperature sensitive, resulting in derepression of the *fad* regulon during growth at elevated temperatures. Table 7 presents the specific activities of the five key β-oxidative enzymes measured in extracts of RS3097, RS3098, and RS3099 grown under various conditions. These data clearly indicate that the temperature-dependent phenotype of strain RS3099 is the result of a thermal induction of the structural genes for the *fad* enzymes. When RS3097 and RS3098 were grown at 25°C, the *fad* enzymes were induced only when oleate was present in the growth medium, whereas these enzymes were expressed constitutively in RS3099 grown at 25°C. However, growth of the *fadR*(Ts) strain RS3097, in the absence of oleate at 37°C, brought about an elevation of each of the *fad* enzyme activities. A similar temperature-dependent elevation was not observed in either RS3098 or RS3099. Varying the temperature at which the enzyme assays were performed had no effect sufficient to explain the temperature-dependent phenotype of strain RS3097 (data not shown). It should be noted, however, that we were unable to obtain reliable crotonase activities at 37°C. This was true for all extracts, and appeared to reflect some lability of this enzyme under these in vitro conditions.

TABLE 7. *Specific activities of the β-oxidative enzymes from extracts of RS3097, RS3098, and RS3099*[a]

Strain	Growth conditions[b]	Oleoyl-CoA[c] synthetase	Palmitoyl-CoA dehydrogenase	Crotonase	β-Hydroxybutyryl-CoA dehydrogenase	Acetoacetyl-CoA thiolase
RS3097 [*fadR*(Ts)]	Acetate, 25°C	0.36	0.5	85	99	11
	Acetate, 37°C	1.15	2.6	230	410	44
	Acetate + oleate, 25°C	1.00	3.2	385	600	65
	Acetate + oleate, 37°C	0.99	3.9	402	558	68
RS3098 (*fadR*$^+$)	Acetate, 25°C	0.15	0.2	108	77	7
	Acetate, 37°C	0.12	0.1	90	119	10
	Acetate + oleate, 25°C	1.52	3.8	548	678	77
	Acetate + oleate, 37°C	1.69	3.8	510	575	85
RS3099 (*fadR*)	Acetate, 25°C	1.83	4.5	360	435	77
	Acetate, 37°C	1.98	4.2	370	515	83
	Acetate + oleate, 25°C	1.96	5.6	424	620	67
	Acetate + oleate, 37°C	1.64	5.0	428	527	41

[a] Enzyme activities are expressed in nanomoles per minute per milligram of protein.
[b] Cells were grown at indicated temperatures in medium M9 supplemented with carbon sources at the following concentrations: potassium acetate, 80 mM; potassium oleate, 5 mM. All cultures contained Brij 58 (5 mg/ml).
[c] CoA, Coenzyme A.

Finally, when various extracts were mixed and the enzymes were assayed at 25 or 37°C, the specific activities remained additive (data not shown). As a whole, these results confirm that the temperature-dependent phenotype exhibited by RS3097 is a result of the altered regulation of *fad* gene expression, and strongly suggest that the *fadR* gene codes for a repressor protein.

The extent of thermal induction in RS3097. When grown on acetate at 37°C, RS3097 is only partially constitutive for the β-oxidation of oleate, and this constitutivity is further inducible by the presence of oleate during growth (Table 6). To determine the extent of thermal induction in RS3097, RS3098, and RS3099, these strains were grown in Trypticase peptone at various temperatures (25 to 42°C), the cells were harvested, and then the rate of oleate oxidation, in vivo, was assayed at 37°C. The results (Fig. 3) illustrate that the ability of RS3097 to oxidize oleate increased continually over the range of culture temperatures examined.

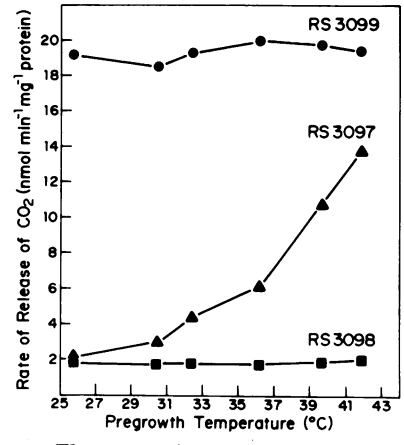

FIG. 3. *The extent of thermal induction in RS3097. RS3097, RS3098, and RS3099 were grown at various temperatures in medium M9 supplemented with 1.0% Trypticase peptone and Brij 58 (5 mg/ml). Cells were harvested, and the rate of oxidation of [1-^{14}C]oleate (5 mM) was determined at 37°C as described in the text.*

DISCUSSION

The studies presented in this paper strongly suggest that the *fadR* gene product is a repressor protein that exerts negative control over the fatty acid degradation system of *E. coli*. Our finding that insertion of Tn*10* into the *fadR* gene results in constitutivity of the *fad* enzymes has important implications for the mechanism of *fad* regulation. Both insertion and spontaneous lesions derepress at least five key enzymes of the β-oxidative cycle; they each endow the cell with the ability to oxidize fatty acids at constitutive levels; the constitutivity in each remains catabolite repressible; and they both extend the range of fatty acids that can serve as sole carbon sources. Moreover, both mutations map at the same location on the *E. coli* chromosome. That the Dec$^+$ phenotype of these *fadR*::Tn*10* strains is indeed the result of the insertion of Tn*10* into the *fadR* gene is indicated by our findings that, in a variety of genetic crosses involving *fadR*::Tn*10* strains, the Tc^r and Dec$^+$ phenotypes remain linked. Genes interrupted by transposons

suffer a complete loss of function (4, 12, 13), and, therefore, in strains harboring insertion mutations in the *fadR* gene, it is reasonable to assume that no functional *fadR* product would be present. If this product is a repressor, then the resulting phenotype would be constitutivity of the *fad* enzymes. If, on the other hand, the *fadR* gene coded for an activator, complete inactivation of the gene would result in non-inducibility. The constitutivity in *fadR*::Tn*10* strains therefore strongly suggests that the *fadR* gene product is a diffusible repressor protein. Further support for this repressor hypothesis comes from the behavior of the Tc^s revertants of *fadR*::Tn*10* mutants: in every case examined, these revertants were Dec^+. In view of the observation that Tn*10* excises predominantly in an imprecise manner (4), it is very likely that many of these Tc^s revertants harbor deletions in the *fadR* gene, although this has not been clearly established here. Deletion of a repressor gene would be expected to lead to constitutivity of the controlled structural genes. Moreover, these results also tend to rule out polarity as an explanation for the Dec^+ phenotype in strain RS3040.

The behavior of *fadR*(Ts) mutants also supports the repressor hypothesis. The strains we have isolated and characterized are constitutive for the enzymes of the *fad* regulon when the cells are grown at an elevated temperature, but remain inducible by long-chain fatty acids at lower temperatures. The mutation responsible for this thermal induction maps at the *fadR* locus. The simplest explanation for this temperature-dependent phenotype is that these strains harbor a thermolabile repressor: at the lower, permissive temperatures, the *fad* regulon is repressed normally, whereas at nonpermissive temperatures, the repressor becomes inactive, resulting in constitutivity.

Our placement of *fadR* at 25.5 min confirms the earlier observations of Vanderwinkel et al. (24) and shows that this putative *fad* regulatory gene is not linked to any of the known *fad* structural genes. We have adopted, as a working model, that the *fadR* gene codes for a repressor protein. Definitive testing of this model must await the construction of appropriate merodiploid strains for the assessment of dominance between various *fadR* alleles, and analysis of putative *fadR* deletions. Such approaches are now possible with the location of *fadR* and its flanking markers clearly established.

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