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Elevated Levels of Glyoxylate Shunt Enzymes in *Escherichia coli* Strains Constitutive for Fatty Acid Degradation

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Mutants of *Escherichia coli* K-12 constitutive for the synthesis of the enzymes of fatty acid degradation (*fadR*) have elevated levels of the glyoxylate shunt enzymes, isocitrate lyase and malate synthase. A temperature-sensitive *fadR* strain has high levels of glyoxylate shunt enzymes when grown at elevated temperatures but has low, inducible levels of glyoxylate shunt enzymes when grown at low temperatures. The increased activity of glyoxylate shunt enzymes does not appear to be due to the degradation of intracellular fatty acids in *fadR* strains or differences in allosteric effectors in *fadR* versus *fadR*$^+$ strains. These studies suggest that the *fadR* gene product may be involved in the regulation of the glyoxylate operon.

The pathway of fatty acid degradation (*fad*) in *Escherichia coli* K-12 has been extensively studied (4, 10, 11, 16). The genes coding for the enzymes of the β-oxidation pathway are located at several sites on the chromosome and comprise a regulon (4, 10). 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 (4, 10, 16). Fatty acids with chain lengths of C_{11} or less can serve as substrates for the *fad* enzymes but cannot induce the synthesis of these enzymes. Thus, only fatty acids longer than C_{11} can be used as a sole carbon source by wild-type strains. Spontaneous mutants constitutive for the synthesis of the *fad* enzymes can be isolated by selecting for growth on the non-inducing fatty acid decanoate as the sole carbon source (4, 10). These mutants harbor lesions in a regulator locus, *fadR* (4), which has been recently mapped at 25.5 min on the revised *E. coli* K-12 linkage map (11, 13). Recent studies suggest that the *fadR* gene codes for a repressor (11).

E. coli degrades fatty acids to acetyl coenzyme A (acetyl-CoA) which, in turn, is mainly catabolized by the tricarboxylic acid cycle (6). However, since with each turn of the tricarboxylic acid cycle two carbon atoms are lost as CO_2, no net assimilation of carbon from acetyl-CoA can occur by this means. Thus, to grow on the acetyl-CoA produced when fatty acids are present as the sole carbon source, a mechanism must be available to replenish the dicarboxylic acids drained from the tricarboxylic acid cycle for cellular biosynthesis (5). This is accomplished in *E. coli* by utilizing the glyoxylate shunt (Fig. 1). The two unique enzymes of the glyoxylate shunt, isocitrate lyase and malate synthase, are induced when acetate or fatty acids serve as the sole carbon source (5). The net effect of the glyoxylate shunt is the formation of 1 mol of dicarboxylic acids from 2 mol of acetyl-CoA (17).

The structural genes for isocitrate lyase, *aceA*, and malate synthase A, *aceB*, map at 89 min on the *E. coli* K-12 linkage map and appear to constitute an operon (1). This operon has been reported to be regulated by an adjacent gene, designated *iclR*. A second malate synthase, malate synthase G, is coded for by the *glc* gene which maps at 64 min (17). In this paper we present evidence which suggests that the synthesis of the glyoxylate shunt enzymes, isocitrate lyase and malate synthase, are regulated by the *fadR* gene.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are shown in Table 1. Preparation of phage stocks and transductions were performed as previously described (11). All strains are derivatives of *E. coli* K-12 selected by growth on decanoate as a sole carbon source. Strain RS3040 is a *fadR* derivative of K-12 which harbors a translocatable tetracycline resistance element, Tn*10*, in its *fadR* gene (11). Strains unable to β-oxidize fatty acids were converted to *fadR* derivatives as follows. Strains K1 (*fad-5*), K19 (*fadE*), and K27 (*fadD*) were transduced to Tc^r with phage P1 *vir* grown on RS3040 (*fadR*::Tn*10*). The *fadR*::Tn*10* derivatives have constitutive levels of the five key *fad* enzymes other than those associated with the original *fad* lesion (data not shown).

The strains defective for malate synthase were produced as follows. Strain SM1021 was a *ppc*$^+$ derivative of DV21AO1 (*aceB glc-1 ppc*) obtained by transduction with phage P1 *vir* grown on K-12 and selected for the ability to grow on D-glucose. Strain SM1022 was a *fadR*::Tn*10* derivative of SM1021 obtained by transduction to Tc^r as described above. Although the *aceB*

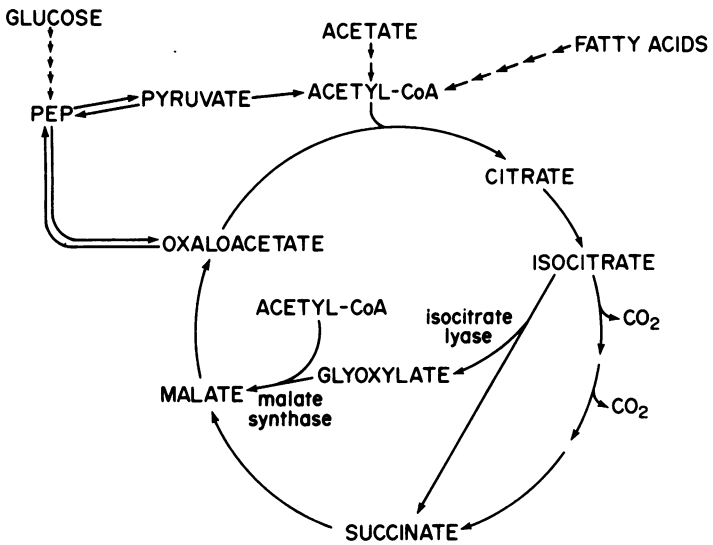

FIG. 1. *Glyoxylate shunt in E. coli and related reactions.*

glc mutants retained relatively high basal malate synthase activities (see Table 5), these strains were unable to grow on acetate as a sole carbon source.

To obtain a variety of *ace* mutants in a K-12 background a *metA* derivative of K-12 was constructed by transducing the transposon Tn*10* into the chromosome near *metA* as follows. Strain AB2569 (*metA*) was transduced to Met^+ Tc^r with P1 *vir* phage grown on a mixed culture of K-12 colonies, each individually resistant to tetracycline due to the insertion of Tn*10* in a different region of the chromosome (3). A P1 *vir* phage stock prepared from this strain was used to transduce AB2569 to Tc^r. Both Met^+ and Met^- transductants were obtained. A Met^- Tc^r transductant was isolated, and a P1 *vir* phage stock was prepared from it. This phage stock was then used to transduce strains K-12 and RS3010, and a Met^- Tc^r (*metA hie*::Tn*10*) transductant of each was isolated. These strains were designated SM6001 and SM6005, respectively. Strains SM6009 (*aceA hie*::Tn*10*) and SM6016 (*aceA hie*::Tn*10 fadR*) were obtained by transducing SM6001 and SM6005, respectively, to Met^+ with phage P1 *vir* grown on strain R4-5 (*aceA*).

Media and growth conditions. Bacteria were routinely incubated in a Gyrotory water bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 37°C. The bacteria were usually grown on medium E (15). Carbon sources and supplements were sterilized separately and added to the culture medium before inoculation. All organic acids were added as the neutralized salts. Acetate was provided at 50 mM final concentration. Succinate and D-glucose were provided at 25 mM final concentration. Fatty acids were suspended in 10% Brij 58, neutralized with potassium hydroxide, sterilized, and added to the medium at a final concentration of 5 mM in the presence of 5 mg of Brij 58 per ml. Tetracycline was added to yield a final concentration of 20 μg/ml. Cell growth was monitored at 540 nm on a Klett-Summerson colorimeter.

Preparation of cell extracts. Bacteria were harvested from mid-log phase cultures (ca. 6.0×10^8 cells/ml), washed three times with ice cold 100 mM potassium phosphate buffer (pH 7.0), and suspended in 1/40 volume of the same buffer. The cells were then disrupted at 4°C in an Aminco French pressure cell at 15,000 lb/in^2 (10^9 dynes/cm^2). The lysate was centrifuged at 27,000 × *g* for 30 min at 4°C, and the resultant supernatant was held at 4°C. Protein content of the extracts was determined by a microbiuret procedure (8) with bovine serum albumin as the standard.

Enzyme assays. The enzymes of the β-oxidation cycle were assayed as previously described (9). Isocitrate lyase and malate synthase were assayed by a modification of the procedures of Dixon and Kornberg (2). Isocitrate lyase activity was determined by mixing 50 μl of cellular extract with 1.0 ml of a freshly prepared reaction mixture containing 6 μmol of $MgCl_2$, 4 μmol of phenyl hydrazine HCl, and 12 μmol of cysteine HCl in 100 mM potassium phosphate buffer (pH 7.0). The increase in absorbance at 324 nm was followed after addition of 8 μmol of trisodium DL-isocitrate. Malate synthase activity was determined by mixing 50 μl of cellular extract with 0.8 ml of a freshly prepared reaction mixture containing 15 μmol of $MgCl_2$ and 0.2 μmol of acetyl-CoA in 0.1 M tris(hydroxymethyl)-aminomethane-hydrochloride buffer (pH 8.0). The decrease in absorbance at 232 nm was monitored after the addition of 10 μmol of sodium glyoxylate. Enzyme reactions were monitored in a Beckman recording spectrophotometer at room temperature. Specific activities are reported as nanomoles of substrate transformed per minute per milligram of protein.

Chemicals. All reagents were used without further purification. Isocitrate, glyoxylic acid, acetyl-CoA, phenylhydrazine HCl, and cysteine HCl were all obtained from Sigma Chemical Co., St. Louis, Mo. All carbon sources were also purchased from Sigma Chemical Co. [1-^{14}C]acetic acid was obtained from New England Nuclear Corp., Boston, Mass. All other chemicals employed were of reagent grade.

RESULTS AND DISCUSSION

Activities of glyoxylate shunt enzymes in *fadR*$^+$ and *fadR* strains. The first indication that the activities of the glyoxylate shunt enzymes might be regulated by the *fadR* gene was noted during studies with the isogenic strains K-12 (*fadR*$^+$), RS3010 (*fadR*), and RS3040 (*fadR*::Tn*10*). When these strains were grown in media containing succinate or malate as the sole carbon source, the activity of isocitrate lyase was approximately tenfold higher in the *fadR* strains, RS3010 and RS3040, than in the *fadR*$^+$ strain, K-12 (Table 2). The activity of malate synthase was approximately threefold greater in the *fadR* strains than in the *fadR*$^+$ strain (Table 2). When grown on acetate or oleate as the sole carbon source, all three strains exhibited comparable levels of isocitrate lyase and malate synthase activities. Interestingly, the activity of isocitrate lyase in the oleate-grown cultures of these three strains was greater than the activity in acetate-grown cultures (Table 2). Growth of these strains on glucose severely repressed the levels of isocitrate lyase and malate synthase activities in all three strains (Table 2). In all other noncatabolite-repressing growth media tested, the *fadR* strains, RS3010 and RS3040, had significantly higher levels of isocitrate lyase and malate synthase activities than the *fadR*$^+$ strain, K-12 (data not shown). Several other *fadR* strains (i.e., 11 independently isolated spontaneous *fadR* strains and 10 independently constructed *fadR*::Tn*10* strains) and their corresponding parental *fadR*$^+$ strains were also examined and, in all cases, the *fadR* strains had greater levels of glyoxylate shunt enzyme activities than the *fadR*$^+$ strains (data not shown). The lesion in all of the *fadR* strains studied was found to map at 25.5 min on the *E. coli* revised chromosome map (data not shown). These results suggest that the synthesis of the glyoxylate shunt enzymes may be derepressed in strains that have defects in their *fadR* gene.

To further substantiate the above hypothesis, the levels of glyoxylate shunt enzyme activities in a temperature-sensitive *fadR* strain were examined. Strain RS3097 is a mutant of *E. coli* K-12 which is inducible for the synthesis of *fad*

TABLE 1. *Bacterial strains*

Strain	Genotype	Source
K-12	Prototrophic	J. Lederberg strain via CGSC[a]
RS3010	*fadR1*	R. Simons et al. (11)
RS3040	*fadR13*::Tn*10*[b]	R. Simons et al. (11)
Ymel	Prototrophic	CGSC
K1	*fad5*	P. Overath strain via CGSC
K1DT	*fad5 fadR13*::Tn*10*	This paper
K19	*fadE*	P. Overath strain via CGSC
K19DT	*fadE fadR13*::Tn*10*	This paper
K27	*fadD*	P. Overath strain via CGSC
K27DT	*fadD fadR13*::Tn*10*	This paper
RS3097	*fadR*(Ts) *bee-101*[b]:: Tn*10*	R. Simons et al. (11)
RS3098	*bee-101*::Tn*10*	R. Simons et al. (11)
RS3099	*fadR1 bee-101*::Tn*10*	R. Simons et al. (11)
SM6001	*metA hie*[b]::Tn*10*	This paper
SM6005	*metA hie*::Tn*10*, *fadR1*	This paper
SM6009	*aceA1 hie*::Tn*10*	This paper
SM6016	*aceA1 fadR1 hie*:: Tn*10*	This paper
SM1021	*aceB6 glc-1 thi-1 relA1 lacZ43*	This paper
SM1022	*aceB6 glc-1 fadR13*:: Tn*10 thi-1 relA1 lacZ43*	This paper
DV21A05	*aceB6 glc-1 ppc-2 thi-1 relA1 lacZ43*	E. Vanderwinkle strain via CGSC
R4-5	*aceA1 metB1 relA1*	H. Kornberg strain via CGSC
AB2569	*metA28 proA2 his-4 argE43 thi-1 lacY1 galK2 xyl-5 mtl-1 tsx-29 supE44*	G. Eggertsson strain via CGSC

[a] CGSC strains obtained from B. Bachmann, Coli Genetic Stock Center, Yale University, New Haven, Conn.

[b] Tn*10* insertions are designated as previously described (11). When an insertion is not within a known gene, the first two letters indicate the numerical position on the *E. coli* linkage map (e.g., *be* corresponds to 25 min and *hi* corresponds to 89 min), and the third letter (*e*) indicates that the insertion is in the *E. coli* chromosome.

TABLE 2. *Specific activities of glyoxylate shunt enzymes from extracts of strains grown on various carbon sources*

Carbon source	Sp act[a]					
	Isocitrate lyase			Malate synthase		
	K-12	RS3010	RS3040	K-12	RS3010	RS3040
Succinate	21	207	185	111	378	361
Malate	31	312	309	111	231	263
Oleate	633	654	651	313	477	558
Dextrose	2	3	5	56	68	76
Acetate	244	312	345	586	601	602

[a] All activities are expressed as nanomoles per minute per milligram of protein. All values are averages of at least two separate determinations.

enzymes at 22°C but constitutive for the synthesis of the *fad* enzymes at 42°C (11). Strains RS3098 and RS3099 are isogenic *fadR*$^+$ and *fadR* strains, respectively. The results in Table 3 show that the activities of isocitrate lyase and malate synthase in the *fadR*(Ts) strain grown on succinate at 42°C are sixfold and threefold higher, respectively, than the activities of these enzymes when this strain is grown at 22°C. Interestingly, the activities of both these enzymes in acetate-grown cultures were slightly greater when cells were grown at 42°C compared to 25°C (Table 3). The activities of isocitrate lyase and malate synthase in strains RS3098 and RS3099 were similar to that described above for the *fadR*$^+$ and *fadR* strains (Table 2) and were not significantly affected by the growth temperature (Table 3). These results suggest that the activity of the glyoxylate shunt enzymes is regulated in the same manner as the *fad* enzymes in the *fadR*(Ts) strain.

Levels of glyoxylate shunt enzymes in *fadR*$^+$ and *fadR fad* mutant strains. Since it was conceivable that high levels of glyoxylate shunt enzymes might be induced in *fadR* strains as a consequence of the endogenous buildup of acetate resulting from the intracellular degradation of fatty acids by the constitutive level of *fad* enzymes in these strains, the level of glyoxylate shunt enzyme activities in *fadR*$^+$ *fad* and *fadR fad* strains was studied. Three different *fad* strains, K1 (*fad-5*), K19 (*fadE*), and K27 (*fadD*) were compared with their isogenic *fadR*::Tn*10* derivatives. Each of these strains was unable to grow on long-chain fatty acids and β-oxidized fatty acids at rates less than 1% that of wild-type strains (Table 4). The results in Table 4 show that the *fadR*::Tn*10* *fad* strains have greater levels of glyoxylate shunt enzyme activities than the parental *fadR*$^+$ *fad* strains. These results suggest intracellular fatty degradation in *fadR* strains is not required for the increase in the activities of the glyoxylate shunt enzymes in these strains. The latter suggestion was further substantiated by the observation that *fadR* and *fadR*$^+$ strains degraded intracellular [1-^{14}C]acetate-labeled lipids at comparable rates (data not shown). Thus, since fatty acid degradation per

TABLE 3. *Specific activities of glyoxylate shunt enzymes in isogenic fadR(Ts), fadR*$^+$*, and fadR strains grown at 22 and 42°C*[a]

Strain	Genotype	Sp act							
		Isocitrate lyase				Malate synthase			
		Succinate		Acetate		Succinate		Acetate	
		22°C	42°C	22°C	42°C	22°C	42°C	22°C	42°C
RS3098	*fadR*$^+$	6	6	248	248	110	168	568	576
RS3097	*fadR*(Ts)	19	127	310	438	145	345	447	650
RS3099	*fadR*	91	144	393	410	344	349	564	633

[a] Cultures grown at indicated temperature. All enzyme assays performed at 22°C. All specific activities represent averages of at least two separate determinations, expressed as nanomoles per minute per milligram of protein.

TABLE 4. *Specific activities of glyoxylate shunt enzymes in fad and fad fadR strains*

Strain	Genotype[a]	Rate of $^{14}CO_2$ released[b] (nmol min^{-1} mg $protein^{-1}$)	Sp act[c]			
			Isocitrate lyase		Malate synthase	
			Succinate	Acetate	Succinate	Acetate
Yme1	*fad*$^+$ *fadR*$^+$	20.2	30	326	115	558
K1	*fad5*	0.13	35	318	118	634
K1DT	*fad5 fadR*::Tn*10*	0.14	227	508	269	649
K19	*fadE*	<0.1	29	369	117	539
K19DT	*fadE fadR*::Tn*10*	<0.1	264	496	282	546
K27	*fadD*	<0.1	45	392	196	599
K27DT	*fadD fadR*::Tn*10*	<0.1	342	501	587	642

[a] Enzymatic defects are as follows: *fad-5*, thiolase, β-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, isomerase; *fadD*, acyl-CoA synthetase; *fadE*, a flavoprotein required by 3-hydroxyacyl-CoA dehydrogenase (4, 10).

[b] Rate of $^{14}CO_2$ release from [1-^{14}C]oleate in vivo as previously described (16).

[c] All specific activities expressed as nanomoles per minute per milligram of protein. All values are averages for at least two separate determinations.

se does not appear to cause the increased activity of glyoxylate shunt enzymes, these results suggest that the *fadR* gene product may be involved in the regulation of the glyoxylate shunt enzymes.

Enzyme-mixing studies on *fadR*$^+$ and *fadR* strains. The high levels of glyoxylate shunt enzyme activities in *fadR* strains could be due to: (i) the presence of an allosteric activator in *fadR* strains that is normally absent in *fadR*$^+$ strains; (ii) the absence of an allosteric inhibitor in *fadR* strains that is normally present in *fadR*$^+$ strains; or (iii) a greater number of enzyme molecules in *fadR* strains. To determine which of these alternatives might explain the high levels of glyoxylate shunt enzyme activities in *fadR* strains, enzyme extracts of *fadR*$^+$ and *fadR* strains were mixed. Crude extracts from strain RS3040 grown on succinate or acetate were mixed with extracts of strain K-12 grown on succinate or acetate, and the activities of isocitrate lyase and malate synthase in the mixed extracts were determined. The results of these experiments indicated that the activities of these enzymes in the mixed extracts were in all cases approximately equivalent to the sum of the activities of unmixed extracts (data not shown). Similar results were observed over a 40-fold range of dilution of enzyme extracts. The mixing studies suggest that the increased level of activity of the glyoxylate shunt enzymes in *fadR* strains is due to an increase in the synthesis of the enzymes and not to an allosteric enhancement of the activities of these enzymes.

Specific activities of glyoxylate shunt enzymes in *aceA* and *aceB glc* strains and isogenic *fadR* derivatives. Elevated levels of glyoxylate shunt enzymes in *fadR* strains could be due to either (i) a greater rate of synthesis of isocitrate lyase and malate synthase coded for by the *aceA* and *aceB glc* genes, respectively, or (ii) the induction of a different isocitrate lyase and malate synthase activity in these strains. To differentiate between these two possibilities, we determined the specific activities of the glyoxylate shunt enzymes in *fadR*$^+$ strains defective for *aceA* or *aceB glc* and in *fadR*::Tn*10* derivatives of these strains. The *fadR* derivatives of *aceA* strains showed no detectable levels of isocitrate lyase when grown on succinate or succinate plus acetate; however, the malate synthase activity of these strains remained threefold higher than that of the *fadR*$^+$ *aceA* parental strain when grown on succinate (Table 5). Similarly, the levels of malate synthase in *fadR aceB glc* strains did not differ from that of the *fadR*$^+$ *aceB glc* parental strain although the isocitrate lyase activity was elevated in the *fadR* derivative after growth on succinate (Table 5). This indicates that *fadR* strains do not induce a unique isocitrate lyase or malate synthase activity.

Both the metabolism of exogenous acetate and the degradation of fatty acids produce acetyl-CoA which must be further metabolized for growth on these substrates as a sole carbon source. Therefore, an association between the degradation of fatty acids and further metabolism of acetyl-CoA seems reasonable. Normally when other anaplerotic mechanisms are available, for example during growth on a glycolytic or gluconeogenic carbon source, the glyoxylate shunt is repressed (5, 6, 13). However, during growth on acetate or fatty acids, the glyoxylate shunt serves as the sole anaplerotic pathway (5). The enzymes of the glyoxylate shunt, isocitrate lyase and malate synthase, are believed to be derepressed by low levels of pyruvate and phosphoenolpyruvate that result when fatty acids or acetate are supplied as the sole carbon source (5). However, there is some evidence that this may not fully account for the regulation of these

TABLE 5. *Specific activities of isocitrate lyase and malate synthase in aceA and aceB glc strains and fadR derivatives*

Strain	Relevant genotype	Sp act[a]					
		Isocitrate lyase			Malate synthase		
		Succinate	Acetate	Succinate + acetate	Succinate	Acetate	Succinate + acetate
K-12	Prototrophic	18	244	70	103	410	186
RS3010	*fadR1*	200	283	247	290	633	324
RS3040	*fadR13*::Tn*10*	181	345	226	267	594	330
SM6009	*aceA hie*::Tn*10*	<1	—[b]	<1	98	—[b]	196
SM6012	*aceA hie*::Tn*10 fadR1*	<1	—	<1	276	—	311
SM1021	*aceB glc-1*	22	—	198	32	—	32
SM1022	*aceB glc-1 fadR13*::Tn*10*	102	—	224	33	—	31

[a] All activities expressed as nanomoles per minute per milligram of protein. All values are averages of at least two separate determinations.

[b] —, No growth on acetate as the sole carbon source.

enzymes (6, 7, 14). This paper implies that this process may be more complex and may be related to fatty acid degradation. Our results suggest that the *fadR* gene product may have some direct effect on the regulation of the glyoxylate operon allowing the induction of the glyoxylate shunt enzymes simultaneously with induction of the enzymes of fatty acid degradation. However, since the regulation of the glyoxylate operon itself is still only poorly understood (1, 7, 14), the mechanism of control by the *fadR* gene product is not yet apparent.

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