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Role of *fadR* and *atoC*(Con) Mutations in Poly(3-Hydroxybutyrate-Co-3-Hydroxyvalerate) Synthesis in Recombinant *pha*$^+$ *Escherichia coli*

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Recombinant *Escherichia coli fadR atoC*(Con) mutants containing the polyhydroxyalkanoate (PHA) biosynthesis genes from *Alcaligenes eutrophus* are able to incorporate significant levels of 3-hydroxyvalerate (3HV) into the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)]. We have used *E. coli fadR* (FadR is a negative regulator of fatty acid oxidation) and *E. coli atoC*(Con) (AtoC is a positive regulator of fatty acid uptake) mutants to demonstrate that either one of these mutations alone can facilitate copolymer synthesis but that 3HV levels in single mutant strains are much lower than in the *fadR atoC*(Con) strain. *E. coli atoC*(Con) mutants were used alone and in conjunction with *atoA* and *atoD* mutants to determine that the function of the *atoC*(Con) mutation is to increase the uptake of propionate and that this uptake is mediated, at least in part, by *atoD*$^+$. Similarly, *E. coli fadR* mutants were used alone and in conjunction with *fadA*, *fadB*, and *fadL* mutants to show that the effect of the *fadR* mutation is dependent on *fadB*$^+$ and *fadA*$^+$ gene products. Strains that were mutant in the *fadB* or *fadA* locus were unable to complement a PHA biosynthesis pathway that was mutant at the *phaA* locus (thiolase), but a strain containing a *fadR* mutation and which was *fadA*$^+$ *fadB*$^+$ was able to complement the *phaA* mutation and incorporated 3HV into P(3HB-co-3HV) to a level of 29 mol%.

Polyhydroxyalkanoates (PHAs) are accumulated as intracellular storage polymers by a wide variety of bacteria, usually in response to metabolic stress. The first PHA discovered was poly-3-hydroxybutyrate (PHB), but many other PHAs have been discovered in recent years. PHAs range in the size of their monomeric units from a 3-carbon compound, 3-hydroxypropionate, to a 12-carbon compound, 3-hydroxydodecanoate. In many cases, PHA polymers are accumulated as copolymers or even terpolymers, with the most noticeable exception being the homopolymer PHB, which is commonly found in many soil microorganisms. The determining factors in the type of polymer that will be produced are the particular bacterial species and the type of carbon source utilized (1, 40).

Commercially, it has been found that these polymers can be used as a biodegradable thermoplastic (5). PHAs consisting of monomeric units having lower carbon numbers, such as PHB, are much less flexible than PHAs containing higher carbon number monomers, such as poly-3-hydroxyoctanoate, which is an elastomer (1). Industrial interest has focused on the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)], which has physical properties similar to those of polypropylene (5). This copolymer can be made in *Alcaligenes eutrophus* by growing the bacteria on a carbon source such as fructose, to facilitate the synthesis of 3-hydroxybutyrate (3HB) units, and another carbon source such as propionate or valerate, to facilitate the synthesis of 3-hydroxyvalerate (3HV) (17).

Three enzymes constitute the *A. eutrophus* PHA biosynthesis pathway, 3-ketothiolase (PhaA), acetoacetyl coenzyme A (CoA) reductase (PhaB), and PHA synthase (PhaC) (1). The 3-ketothiolase catalyzes the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA, which is reduced to 3HB-CoA via the action of the acetoacetyl-CoA reductase. The PHA synthase polymerizes 3HB-CoA to form PHB. In the case of 3HV formation, it is thought that the ketothiolase catalyzes the condensation of propionyl-CoA and acetyl-CoA to 3-ketovaleryl-CoA followed by reduction to 3HV-CoA by the acetoacetyl-CoA reductase. The PHA synthase adds this compound to the growing polymer. Evidence for this scenario is found in the substrate specificities of the three PHA enzymes (12, 14, 15), the fact that extracellular propionate is rapidly incorporated into propionyl-CoA (8), and the demonstration that *A. eutrophus* mutants having higher intracellular levels of propionyl-CoA are able to synthesize 3HV without the addition of propionate (39).

The *A. eutrophus* PHA biosynthesis genes have been cloned and introduced into *Escherichia coli* (31, 32, 35, 38), where the genes are well expressed. However, these strains are not able to produce P(3HB-co-3HV) when propionate is added to the feedstock in a manner analogous to the procedure used for *A. eutrophus* and other bacteria. This limitation can be circumvented through the use of *E. coli* K-12 strains that are mutant at the *fadR* and *atoC* loci. Recombinant *pha*$^+$ *E. coli* K-12 *fadR atoC*(Con) strains behave much like *A. eutrophus* and other copolymer-producing strains in that the levels of 3HV in the P(3HB-co-3HV) can be elevated by increasing the propionate levels but too much propionate can be toxic, causing lower total polymer levels (37).

The functions of both *fadR*$^+$ and *atoC*$^+$ have been well documented in the literature, and both genes have been cloned and characterized (6, 7, 16, 19, 27). The *fadR* gene encodes a protein that represses the transcription of genes necessary for long-chain fatty acid oxidation (16, 36) and the glyoxylate pathway (22, 24), induces transcription of genes necessary for fatty acid biosynthesis (16, 21), and is necessary for unsaturated fatty acid biosynthesis (28). The *atoC* gene encodes a protein that is a positive activator of genes necessary for medium-chain fatty acid utilization (ATO system) (19, 29). Thus, a mutation

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TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source or derivation[a]
K-12	Wild type	Our collection
LS5218	*fadR601 atoC*(Con)*2*	B. J. Bachmann
LS6590	*fadB64*	P. N. Black
LS6591	*fadA30*	P. N. Black
LJ14	*fadR601 atoC*(Con)*512 atoD514*	B. J. Bachmann
LJ32	*fadR601 atoC*(Con)*512 atoD32*	B. J. Bachmann
RS3242	*trpE61* Tn*5dad* R1, *trpA62 zef-117*::Tn*10*	B. J. Bachmann
RS3032	Lambda *purB58*, *fadR613*::Tn*10*	B. J. Bachmann
CAG18516	MG1655 *zef-3114*::Tn*10kan*	C. A. Gross
CAG18544	MG1655 *fadR3115*::Tn*10*	C. A. Gross
PN109	Δ*fadL fadR*	P. N. Black
JMU170	*fadR*::Tn*10*	P1(RS3032) × K-12
JMU171	*fadR*::Tn*10 atoC*(Con)	P1(RS3032) LS518
JMU172	*zef-3114*::Tn*10kan atoC*(Con)	P1(CAG18516) × JMU171
JMU173	*zef-117*::Tn*10 atoC*(Con)	P1(RS3242) × JMU172
JMU187	*fadR*::Tn*10kan atoC*(Con)*512 atoA514*	P1(CAG18544) × LJ14
JMU188	*fadR*::Tn*10kan atoC*(Con)*512 atoD32*	P1(CGA18544) × LJ32
JMU192	*fadR*::Tn*10* Δ*fadL*	P1(RS3032) × pN109
JMU193	*fadR*::Tn*10 fadB64*	P1(RS3032) × LS6590
JMU194	*fadR*::Tn*10 fadA30*	P1(RS3032) × LS6591

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in *fadR* derepresses transcription of the genes involved in fatty acid oxidation, whereas a constitutive mutation in *atoC*, *atoC* (Con), activates transcription of the ATO genes. The mode of action of these mutations with regard to P(3HB-co-3HV) synthesis in recombinant pha^+ *E. coli* has not been studied, nor has it been determined whether either of these genes can act independently to facilitate P(3HB-co-3HV) synthesis in *E. coli*. This paper reports such studies and indicates that both mutations are able to mediate the incorporation of 3HV into P(3HB-co-3HV). Our results suggest that the primary mode of action of the *atoC*(Con) mutation is to increase the uptake of propionate into the cell and that the primary mode of action of the *fadR* mutation is to increase the levels of fatty acid oxidation enzymes which complement the function of the *phaA* gene.

MATERIALS AND METHODS

Growth of bacterial strains and phages. The bacterial strains used in this study are described in Table 1. All strains are either *E. coli* K-12 or derivatives thereof. Cultures were routinely propagated in Luria-Bertani medium or M9 minimal medium (25) with antibiotics (if necessary) at the specified concentrations (25).

For experiments involving PHB and P(3HB-co-3HV) production, the bacterial strains were inoculated into 3-ml Luria-Bertani tube cultures and grown overnight at 30°C at 225 rpm in an orbital incubator-shaker. A 500-μl portion of the overnight culture was inoculated in 50 ml of Luria-Bertani medium containing antibiotic (if necessary) and grown at 37°C and 225 rpm until the optical density at 600 nm was between 0.8 and 1.2. A 5-ml portion of the culture was removed and pelleted by centrifugation at 3,000 rpm for 5 min in a swinging-bucket rotor (Heraeus Varifuge RF; Baxter Scientific, Columbia, Md.). The supernatant was discarded, and the pellet was resuspended in 5 ml of sterile 0.85% (wt/vol) NaCl. A 500-μl portion of this suspension was inoculated into 50 ml of M9 minimal medium containing antibiotic, 1% (wt/vol) glucose, and 10 mM propionate. The culture was grown at 37°C and 225 rpm. Cultures were harvested for analysis at 24 h postinoculation unless otherwise specified. Alterations of these growth conditions for individual experiments are as described in the text.

Transduction with phage P1*vir* was done as described elsewhere (26).

Plasmid construction. Standard procedures were used for isolation, cleavage, ligation, T4 DNA polymerase fill-in reactions, and electroporation of plasmid DNA (25). Plasmid pJM9131, containing the PHA biosynthesis genes, has been described previously (18, 45).

Plasmid pJM9351 was made as follows. The PHA biosynthesis pathway from *A. eutrophus* was excised from plasmid pSB20 (38) by first cutting with *Eco*RI followed by partial digestion with *Kpn*I. A 5.2-kb fragment, containing the PHA biosynthesis pathway, was ligated into pTZ-18U (United States Biochemical, Cleveland, Ohio) that had been digested with *Eco*RI and *Kpn*I. The plasmid was named pTZ-18U/PHB. Plasmid pTZ-18U/PHB was linearized with *Bam*HI and then partially digested with *Pst*I. A 2.7-kb fragment containing the PHA synthase gene (*phaC*) was cloned into pSP73 (Promega, Madison, Wis.) that had been digested with *Bam*HI and *Pst*I. This plasmid was digested with *Xho*I and *Eco*RI to release the same 2.7-kb fragment and was ligated into pGEM7f (Promega) that had been cut with *Xho*I and *Eco*RI. This plasmid, pJM8906, allowed *phaC* to be excised on a *Hin*dIII fragment. To obtain the acetoacetyl-CoA reductase gene (*phaB*), pTZ-18U/PHB was digested with *Sph*I and *Bam*HI and then exonuclease III was used to unidirectionally delete the PHA biosynthesis genes to approximately base 3890 on the published sequence (18); this was followed by religation. The plasmid, named pJM9005, contained *phaB* and an upstream *Hin*dIII site approximately 60 bases in front of the start of the *phaB* coding region. To make pJM9351, the PHA synthase gene (with promoter) was excised from pJM8906 and cloned into the *Hin*dIII site of pJM9005 with transcription of *phaC* and *phaB* occurring in the same direction.

Measurement of propionate uptake. Bacterial cells grown overnight in M9 minimal medium containing 1% (wt/vol) glucose and 10 mM propionate were pelleted and suspended in 0.85% (wt/vol) NaCl. Washed cell suspensions were then subcultured at low cell density (inoculum size, 1% of final volume) into M9 minimal medium containing 1% glucose and unlabeled propionate. Cells were grown at 37°C to an optical density at 600 nm of 0.8 to 1.0. The cells were harvested by centrifugation, washed twice with carbon-free M9 minimal medium, and resuspended in same medium to give an optical density at 600 nm of approximately 2.0. Cell suspensions (0.5 ml) in test tubes were preincubated at 37°C for 10 min prior to the addition of labeled propionate. The uptake reactions were started by addition of 15 nmol of [1-^{14}C]propionate (NEC-093H, 51 mCi/mmol; Dupont NEN, Wilmington, Del.) to 0.5 ml of the cell suspension. Samples (4 μl) were withdrawn at intervals, filtered through membrane filters (pore size, 0.4 μm; Millipore Corp., Bedford, Mass.), and rinsed three times with 3 ml of carbon-free M9 minimal medium (9 ml total). The filters were dried at room temperature, and the radioactivity was measured with a scintillation counter (Beckman LS5000 TA) by using 4 ml of Scintiverse II (Fisher Scientific, Pittsburgh, Pa.). The nonspecific absorption of radioactivity was assessed by adding labeled propionate in M9 minimal medium without cell suspensions. The transport activity was expressed as nanomoles of propionate per minute per milligram of dry cell weight.

PHB and P(3HB-co-3HV) assay. Quantitative determination of PHB and P(3HB-co-3HV) was performed by gas-chromatographic analysis of extracts obtained from methanolysis of lyophilized cells (37). PHB amounts were calculated from a standard curve by using known quantities of PHB or P(3HB-co-3HV).

Enzyme assays. A 10-ml sample of the bacterial culture was pelleted by centrifugation and resuspended in 500 ml of breaking buffer (20 mM potassium phosphate buffer [pH 7.2], 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 M glycerol). The cell suspension was then disrupted by sonication with four 15-s bursts, using a microtip at maximum setting (sonic dismembrator model 300; Fisher Scientific). The crude extracts were centrifuged in a microcentrifuge for 5 min to sediment cell debris. The supernatants were removed and subjected to enzyme assays. Acetate kinase and phosphotransacetylase assays were conducted

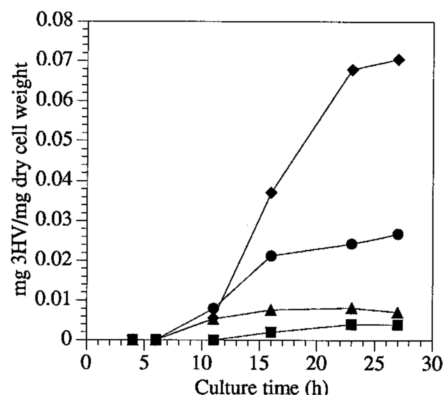

FIG. 1. 3HV incorporation in *E. coli* K-12 (■), *E. coli* JMU170 *fadR* (●), *E. coli* JMU173 *atoC*(Con) (▲), and *E. coli* LS5218 *fadR atoC*(Con) (◆).

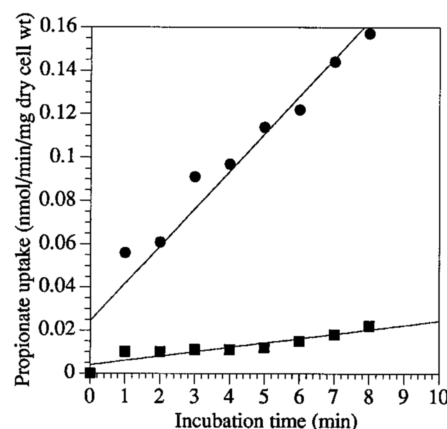

FIG. 2. Propionate uptake in *E. coli* K-12 (■) and *E. coli* JMU173 *atoC*(Con) (●).

by the method of Brown et al. (4). Acetyl-CoA and propionyl-CoA synthetase assays were performed as described by Krahenbuhl and Brass (20).

Protein assay. The protein content in the supernatants used for enzymatic analysis was measured with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

Dry weight analysis. Samples (5 ml) were removed from cultures and placed into preweighed borosilicate glass culture tubes (16 by 100 mm). The cell was pelleted by centrifugation at 3,000 × *g* for 15 min and washed with deionized water. The pellet was then allowed to dry for several days at 55°C and then weighed.

Chemicals and reagents. All chemicals were of the highest quality and were obtained from Sigma Chemical Co., St. Louis, Mo., or from United States Biochemicals. Restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Kanamycin Resistance GenBlock was purchased from Pharmacia LKB Biotechnology, Piscataway, N.J. Agarose was Seakem GTG agarose from Marine Colloids Div., FMC Corp., Rockland, Maine. [1-^{14}C]acetic acid, sodium salt (catalog no. NEC-084H), and [1-^{14}C]propionic acid, sodium salt (catalog no. NEC-093H), were purchased from Dupont NEN. PHB was obtained from Aldrich Chemical Co., Madison, Wis.

RESULTS

Analysis of 3HV production in mutant strains. To study the possibility that *fadR* and *atoC*(Con) mutants independently mediate 3HV incorporation, we made *pha*$^+$ *fadR* (JMU170) and *pha*$^+$ *atoC*(Con) (JMU173) strains and compared them with a *pha*$^+$ *fadR atoC*(Con) (LS5218) and a *pha*$^+$ (K-12) strain as to the type of PHA made during growth in minimal medium containing glucose and propionate (Fig. 1). The results of the time course study indicate that both single-mutant strains are able to independently mediate an increase in 3HV incorporation into P(3HB-co-3HV) with respect to the *pha*$^+$ *E. coli* (K-12) strain. The *fadR* mutant accumulates approximately three times more 3HV than the *atoC*(Con) mutant does. However, the double mutant, *fadR atoC*(Con), was considerably better than either of the single mutants, accumulating 3HV to levels that were much higher than the level of 3HV that accumulated as a result of single mutations [3HV due to *fadR* plus 3HV due to *atoC*(Con)]. After 27 h of growth, the 3HV content for each strain was 2 mol% for K-12, 5 mol% for the *fadR* mutant, 6 mol% for the *atoC*(Con) mutant, and 20 mol% for the *fadR atoC*(Con) mutant. The *atoC*(Con) mutant produced significantly less 3HB than the other strains did, resulting in a moles percent value that was artificially high.

Analysis of propionate uptake. The *atoC* gene product is an activator that is required for the synthesis or activation of the *atoDAB*-encoded enzymes (ATO enzymes) which function in the transport and metabolism of short-chain fatty acid such as butyrate or valerate (19, 29). We noticed that the *atoC*(Con) mutant (JMU173) grew more slowly and accumulated less polymer than the otherwise isogenic strain did. Thus, it is possible that constitutive levels of ATO enzymes in the *atoC* (Con) mutant cause it to accumulate higher levels of intracellular propionate, which is toxic to the cells. To confirm this possibility, we examined the propionate uptake in K-12 and JMU173 (Fig. 2). Transport activities were determined by the initial rate of uptake, with [1-^{14}C]propionate as the substrate. In both cases, the rate of uptake was essentially linear, with the *atoC*(Con) mutant exhibiting an approximately 10-fold-higher uptake rate than the nonmutant strain (0.017 versus 0.0017 nmol of propionate min^{-1} mg of dry cell weight^{-1}).

Analysis of ATO gene contributions to propionate uptake. The presence of the ATO enzymes acetoacetyl-CoA transferase and thiolase II is required for growth of *E. coli* on short-chain fatty acids. Two genes, *atoD* and *atoA*, encode the two subunits of acetoacetyl-CoA transferase, and *atoB* encodes thiolase II (30). Acetoacetyl-CoA transferase is associated with the membrane and is responsible for short-chain fatty acid transport (10). To determine which of the ATO proteins is responsible for propionate uptake, we made *atoC*(Con) *atoA* (JMU187) and *atoC*(Con) *atoD* (JMU188) mutants (containing pJM9131) and examined their effect on propionate uptake (Table 2). The *atoC*(Con) *atoD* mutant did not take up propionate nearly as well as the *atoC*(Con) strain, whereas the *atoC*(Con) *atoA* mutant did. This finding suggests that *atoD* is responsible for propionate uptake. A direct relationship between propionate uptake and 3HV incorporation was observed in this strain. *atoC*(Con) *atoA* and *atoC*(Con) mutants produced 3HV to 10 mol% of the total polymer. However, the

TABLE 2. Effect of *ato* mutations on [^{14}C]propionate uptake and 3HV content

Strain	Relevant genotype	Specific uptake[a]	Amt (mg/mg of DCW) of[b]:		Mol% of 3HV
			3HB	3HV	
K-12		0.0022	0.293	0.003	1.0
JMU173	*atoC*(Con)	0.0256	0.225	0.025	10.0
JMU187	*atoC*(Con) *atoA*	0.0122	0.181	0.029	13.2
JMU188	*atoC*(Con) *atoD*	0.0022	0.163	0.007	4.1

[a] Specific uptake is expressed in nanomoles per minute per milligram of protein.
[b] DCW, dry cell weight.

TABLE 3. Effect of *fad* mutations on 3HV content

Strain	Relevant genotype	Amt (mg/mg of DCW)[a] of:		Mol% of 3HV
		3HB	3HV	
JMU170(pJM9131)	*fadR*	0.110	0.019	14.7
JMU192(pJM9131)	*fadR fadL*	0.147	0.029	16.7
JMU193(pJM9131)	*fadR fadB*	0.046	0.001	2.2
JMU194(pJM9131)	*fadR fadA*	0.204	0.011	4.9

[a] DCW, dry cell weight.

atoC(Con) *atoD* mutant strain exhibited a much lower 3HV content of 4.1 mol%, a two- to threefold reduction in 3HV produced per cell weight. 3HB levels were approximately the same in the three *ato* mutant strains.

Effect of *fadR* on propionate uptake and acetyl-CoA synthesis enzymes. Maloy and Nunn have shown that a mutation in the *fadR* gene increases the uptake of acetate fivefold but does not alter the activity of the primary enzymes of acetyl-CoA synthesis from acetate in *E. coli*, i.e., acetate kinase (Ack) and phosphotransacetylase (Pta) (24). Because we have shown that AckA and Pta mediate the conversion of propionate to propionyl-CoA and subsequent formation of 3HV (34) and because we have already seen that an increase in propionate uptake can significantly affect 3HV incorporation, we analyzed these activities in *E. coli* K-12(pJM9131) and *E. coli* JMU170 *fadR*(pJM9131). We found that there was no significant difference in acetate kinase, phosphotransacetylase, acetyl-CoA synthetase, and propionyl-CoA synthetase activities. Nor was there any fundamental difference in propionate uptake between the two strains (data not shown).

Effect of FAO genes on 3HV incorporation. Although the *fadR* gene product is a multifunctional regulator, it was first discovered and appears to be most significant as a regulator of fatty acid oxidation (27). Therefore, it seemed likely that the fatty acid oxidation (*fad*) genes may play a role in 3HV formation. Of these, the multienzyme complex encoded by *fadA* and *fadB* and the protein encoded by *fadL* seemed the most likely candidates. The *fadAB* gene product has thiolase, enoyl-CoA hydrase, 3-hydroxyacyl-CoA dehydrogenase, hydroxyacyl-CoA epimerase, and enoyl-CoA isomerase activities (2, 33), while the *fadL* gene product functions in transport of long-chain fatty acids (3). The appropriate strains were constructed and used to test this hypothesis in growth studies (Table 3). These results indicated that a mutation in *fadL* has no apparent effect on P(3HB-co-3HV) synthesis. In fact, both 3HB and 3HV levels were slightly higher in this mutant strain (JMU192) than in JMU170 (*fadR*). In contrast to JMU192, the *fadR fadB* mutant (JMU193) exhibited a large decrease in 3HV incorporation and total polymer production. The *fadR fadA* mutant (JMU194) also accumulated less 3HV into copolymer but produced considerably more 3HB than did JMU193.

Complementation of a *phaA* mutation. The data in Table 3 indicate that the *fadAB*$^+$ gene product is instrumental in 3HV incorporation into copolymer. This is consistent with the fact that one of the enzyme activities found in this multienzyme complex is a thiolase, which may have substrate specificity broad enough to allow for the production of 3-ketovaleryl-CoA from acetyl-CoA and propionyl-CoA. To determine if this is the case, a plasmid containing the PHA synthase and acetoacetyl-CoA reductase genes (*phaC* and *phaB*) but lacking the 3-ketothiolase gene (*phaA*) was constructed and put into the same strains used in the preceding experiment. The strains were grown in 1% (wt/vol) glucose or in 1% (wt/vol) glucose containing 10 mM propionate to either deter or facilitate, respectively, 3HV incorporation (Table 4). Total polymer levels were quite low, with the highest being approximately 6% of the dry cell weight. None of the strains grown in glucose exhibited any 3HV incorporation, and only the *fadR* mutant (JMU170) produced any 3HB (4% of dry cell weight). All of the strains grown in glucose-containing propionate exhibited polymer formation, but only the *fadR* mutant accumulated PHA to any significant level (5.7% of dry cell weight). The 3HV content of this polymer was 29 mol%. These results support the contention that the *fadAB* gene product is instrumental in the synthesis of 3HV in *E. coli fadR* mutants grown on glucose containing propionate.

TABLE 4. Effect of *fad* mutations on 3HV production in a Δ*phbA* mutant

Strain	Relevant genotype	3HV production with carbon source:			
		Glucose		Glucose plus propionate	
		Total PHA[a]	Mol% of 3HV	Total PHA	Mol% of 3HV
K-12(pJM9351)		ND[b]	ND	ND	ND
JMU170(pJM9351)	*fadR*	0.040	ND	0.057	29.0
JMU193(pJM9351)	*fadR fadB*	ND	ND	0.004	ND
JMU194(pJM9351)	*fadR fadA*	ND	ND	0.003	ND

[a] Expressed in milligrams per milligram of dry cell weight.
[b] ND, none detected.

DISCUSSION

E. coli K-12 strains harboring *pha*$^+$ plasmids do not produce 3HV to levels above 2 mol%, even when grown in the presence of glucose and propionate to stimulate intracellular propionyl-CoA levels. However, if *fadR* and *atoC*(Con) mutations are introduced into the strain, P(3HB-co-3HV) copolymer containing as much as 50 mol% 3HV can be produced (37). To elucidate the action of these two mutations, we drew from a large body of knowledge concerning these two genes and the genes that they regulate (27).

Growth of *E. coli* on short-chain fatty acids (C_4 to C_6) requires the expression of *atoD*$^+$, *atoA*$^+$, and *atoB*$^+$ genes as well as *fadB*$^+$ and *fadE*$^+$. The *atoA* and *atoD* gene product, acetoacetyl-CoA transferase, appears to play a major role in the transport of acetoacetate and butyrate (10, 11). Therefore, it was not surprising that the propionate uptake rate in *atoC* (Con) mutants was approximately 10-fold higher than in nonmutant cells. Presumably, the specificity of the uptake system is broad enough to allow for a significant amount of propionate uptake. This is supported by the fact that, in general, fatty acid oxidation enzymes and fatty acid biosynthesis enzymes are able to accept a wide range of substrates (24).

Propionate uptake was not significantly affected by a mutation in the *atoA* gene but was drastically decreased by a mutation in the *atoD* gene. These two genes code for the subunits of the acetoacetyl-CoA transferase. Theoretically, it is possible that the binding site for propionate resides on the subunit encoded by *atoD*, but this has not been shown.

Determination of the role of the *fadR* mutation on 3HV incorporation was problematic because of the high degree of pleiotropism exhibited by this mutation (16, 23, 28, 36). Maloy and Nunn showed that the *fadR* mutations stimulate the uptake of acetate but do not stimulate the expression of the enzymes (acetate kinase and phosphotransacetylase) that me-

tabolize acetate (24). Despite this, we believed that it was important to reexamine the possibility that $ackA^+$ and pta^+ are regulated by the $fadR^+$ gene product, because we have shown that these enzymes are necessary for efficient 3HV production (34) and because it is well known that one of the functions of the $fadR^+$ gene product is to stimulate transcription of fatty acid oxidation genes (36). We analyzed the levels of these two enzymes and total acetyl-CoA synthetase and propionyl-CoA synthetase in *fadR* and $fadR^+$ strains and found no noticeable difference, confirming the results of Maloy and Nunn (24).

We also examined the possibility that the *fadR* mutation stimulates propionate uptake, since it is known that *fadR* mutations result in a fivefold increase in acetate uptake (24). We found that propionate uptake was only marginally stimulated in *fadR* mutant strains in comparison with $fadR^+$ strains. This seems to be a reasonable result if the propionate is taken up by the acetate uptake system previously reported (24) but at a lower V_{max}.

The most substantial contribution to 3HV incorporation is made by the cellular 3-ketoacyl-CoA thiolase activity of the $fadA^+$ and $fadB^+$ gene product. Our experiments showed that a *fadR E. coli* mutant containing a plasmid that possessed a *phaC-ΔphaA-phaB* pathway (thiolase deleted) was able to synthesize both 3HB and 3HV, but the introduction of a mutation in either *fadA* or *fadB* removed the ability to make measurable levels of 3HV. The substrate specificity of the cellular 3-ketoacyl thiolase has been measured, and it has been found to have no more than 5 to 10% of the activity on acetoacetyl-CoA that it has on the preferred substrate, 3-ketodecanoyl-CoA (2, 9, 33). Substrate specificities for propionyl-CoA and acetyl-CoA were not measured but are presumably even lower. These data seem to argue against the possibility that the cellular 3-ketoacyl thiolase complements the PHA 3-ketothiolase. On the other hand, both reactions occur via the ping-pong mechanism (41–43), and Yang et al. have found that the active site located at Cys-91 of the *E. coli* 3-ketoacyl-CoA thiolase is similar to the active site located at Cys-89 of the *Zoogloea ramigera* acetoacetyl-CoA thiolase (42, 44). Moreover, their data indicate that the *Z. ramigera* acetoacetyl-CoA thiolase will also bind medium-chain 3-ketoacyl-CoA derivatives as well as its preferred substrate. Haywood et al., employing an in vitro PHA synthesis system and purified enzymes, have determined that either of the two 3-ketoacyl-CoA thiolases found in *A. eutrophus* will function in PHA synthesis (12). One of these enzymes has specificity only for C_4 and C_5 substrates and is likely to be the enzyme responsible for PHB synthesis, while the other enzyme has substrate specificity in the range of C_4 to C_{10} and is thought to be the cellular 3-ketoacyl-CoA thiolase (43). These data, taken together with our data, suggest that the cellular 3-ketoacyl-CoA thiolase can bind intermediates of P(3HB-co-3HV) metabolism and function in copolymer synthesis.

Because the *fadR* and *atoC*(Con) mutations facilitate 3HV incorporation via different but complementary mechanisms, the coalescence of these mutations might be expected to mediate 3HV production to levels greater than those resulting from the sum of the independent actions. This was the case. Under standard conditions (M9 minimal medium containing 1% [wt/vol] glucose and 10 mM propionate), we have rarely detected 3HV incorporations above 7 mol% for a *fadR* mutant and 6 mol% for an *atoC*(Con) mutant. However, we have seen 3HV incorporation as high as 28% for a *fadR atoC*(Con) mutant. We estimate that under normal conditions, the amount of 3HV made by a *fadR atoC*(Con) mutant is two to three times the amount made by adding the 3HV produced individually from a *fadR* mutant and an *atoC*(Con) mutant. This estimate also holds true for the uptake of propionate in *fadR*, *atoC*(Con), and *fadR atoC*(Con) mutants. In general, the uptake of propionate in *fadR atoC*(Con) mutants is two to three times the amount of propionate uptake obtained by adding the individual contributions of the *fadR* and *atoC*(Con) mutations.

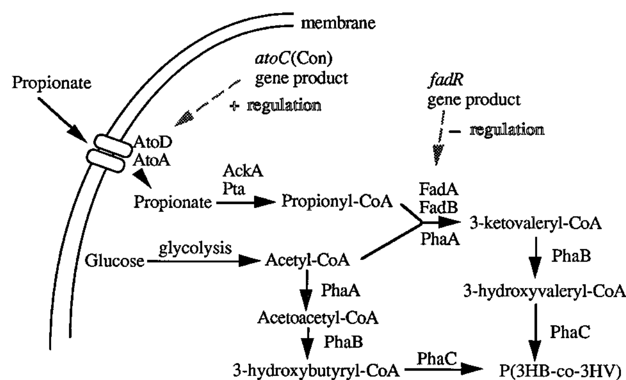

FIG. 3. Model of P(3HB-co-3HV) synthesis in pha^+ *E. coli.*

The data in this paper combined with data from previous research (34) allow us to propose a preliminary model of the mechanism of P(3HB-co-3HV) synthesis in *E. coli* in which two 3HV production pathways are operative (Fig. 3). The first, which has been suggested previously (8, 12, 14, 15), involves the condensation of acetyl-CoA and propionyl-CoA to form 3-ketovaleryl-CoA, a process mediated by the 3-ketothiolase (PhaA). This compound is then reduced and incorporated into the growing polymer via the action of the acetoacetyl-CoA reductase (PhaB) and PHA synthase (PhaC). Our data support this scenario in that *fadR fadB* $phaA^+$ and *fadR fadA* $phaA^+$ strains are able to produce 3HV to between 2 and 5 mol% (Table 3), whereas *fadR fadB phaA* and *fadR fadA phaA* strains produce no detectable 3HV. In the second pathway for 3HV production, propionate is taken up by the ATO system, mediated by the $atoD^+$ gene product, and controlled by the $atoC^+$ gene product. Upon entry into the cell, the propionate is converted to propionyl-CoA by the sequential action of acetate kinase and phosphotransacetylase, which are constitutively expressed. Propionyl-CoA is then condensed with acetyl-CoA by the 3-ketoacyl-CoA thiolase activity of the $fadAB^+$ gene product, which has been derepressed by the action of a mutation in the *fadR* regulatory gene. 3-Ketovaleryl-CoA formed in this manner is incorporated into polymer as described above.

This model leads to several interesting possibilities. It may be possible, by modifying and regulating bacterial fatty acid oxidation and fatty acid biosynthesis genes, to modify PHA polymers produced by bacterial species. This is particularly true if PHA synthases with substrate specificities broader than the *A. eutrophus* PHA synthase are used. In similar experiments, it may also be possible to modify and regulate plant fatty acid oxidation and fatty acid biosynthesis genes to alter PHAs accumulated in plant cells. This is suggested by the fact that eukaryotic fatty acid oxidation genes have been shown to be very similar to their prokaryotic counterparts (6).

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