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ato Operon: a Highly Inducible System for Acetoacetate and Butyrate Degradation in *Escherichia coli*

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1. Growth of *Escherichia coli* in the presence of acetoacetate as inducer stimulates the rate of synthesis of two enzymes, an acetoacetyl-CoA : acetate CoA-transferase and a thiolase by a factor of 3000. The combined action of these enzymes allows for the degradation of acetoacetate to acetate and acetyl-CoA.

2. The structural genes for these enzymes, called *atoA* (CoA-transferase) and *atoB* (thiolase), and a regulatory gene, *atoC*, are closely linked, forming an operon (*ato* operon) with the order *his* . . . *atoCAB* . . . *nalA* on the bacterial chromosome.

3. The regulatory gene *atoC* is defined by a spontaneous mutation to the $atoC^c$ state which allows *E. coli* to grow on butyrate and to synthesize the enzymes of the *ato* operon constitutively. This mutation can only be selected for when the enzymes of the C-3 oxidation pathway are themselves formed constitutively. The $atoC^c$ state is required for butyrate degradation because this acid does not serve as inducer of the *ato* operon but requires the CoA-transferase for activation.

4. The evidence so far available indicates that the $atoC^c$ mutation is *trans*-dominant over the inducible $atoC^+$ state suggesting a positive mechanism of control.

5. The uptake of butyrate is reduced or completely suppressed when mutational lesions prevent the further metabolism of this substrate.

The synthesis of the enzymes of the C-3 oxidation pathway in *Escherichia coli* is induced by long-chain-length fatty acids with more than twelve carbon atoms [1–5]. Spontaneous mutants of the wild type obtained by selection on decanoate as sole carbon source were able to grow on fatty acids with more than eight carbon atoms and had acquired at the same time the ability for constitutive synthesis of a number of enzymes involved in fatty acid degradation [4]. This mutation was shown to be necessary for the occurrence of secondary mutations which allowed the cell to use butyrate as sole carbon source. Two classes of such mutants could be distinguished by their enzymatic and growth properties, one of which is the subject of the present paper. A representative strain (*dec-16 but-1*) of this class contained a new highly active thiolase and had at the same time a reduced ability to grow on medium and long-chain-length fatty acids. It was argued that this mutant must have gained a mechanism for the activation of butyrate. Whereas this prediction is confirmed by the experiments presented below, an alternative, more involved hypothesis [4] for the appearance of the thiolase activity, namely the mutational alteration of the chain-length specificity of this enzyme, can now be rejected. Furthermore, it was shown by genetic experiments that the enzymes of fatty acid degradation are required for growth to occur on butyrate, thereby questioning the physiological importance of butyrate degradation *via* 3-ethylmalate [6]. The requirement for two mutational steps for the ability of *E. coli* to grow on butyrate has recently been confirmed by Salanitro and Wegener [7, 8].

Various aspects of the induction and the properties of the enzymes of fatty acid degradation in *E. coli* have also been studied in the laboratories of Wakil [9, 10] and Ailhaud [11–13]. An important observation for the present study was the presence of a butyryl-CoA : acetate CoA-transferase activity in a butyrate-degrading mutant by Vanderwinkel

Abbreviations. $C_{12:0}$, $C_{10:0}$, $C_{4:0}$ designate dodecanoate, decanoate, and butyrate, respectively.

Enzymes. Enoyl-CoA hydrase or L-3-hydroxyacyl-CoA hydrolyase (EC 4.2.1.17); acyl-CoA synthetase (EC 6.2.1.3); 3-hydroxyacyl-CoA dehydrogenase or L-3-hydroxyacyl-CoA : NAD oxido reductase (EC 1.1.1.35); thiolase or acetyl-CoA : acetyl-CoA *C*-acetyltransferase (EC 2.3.1.9); butyryl-CoA or acetoacetyl-CoA : acetate CoA-transferase (EC 2.8.3.–); phosphotransacetylase or acetyl-CoA : orthophosphate acetyltransferase (EC 2.3.1.8).

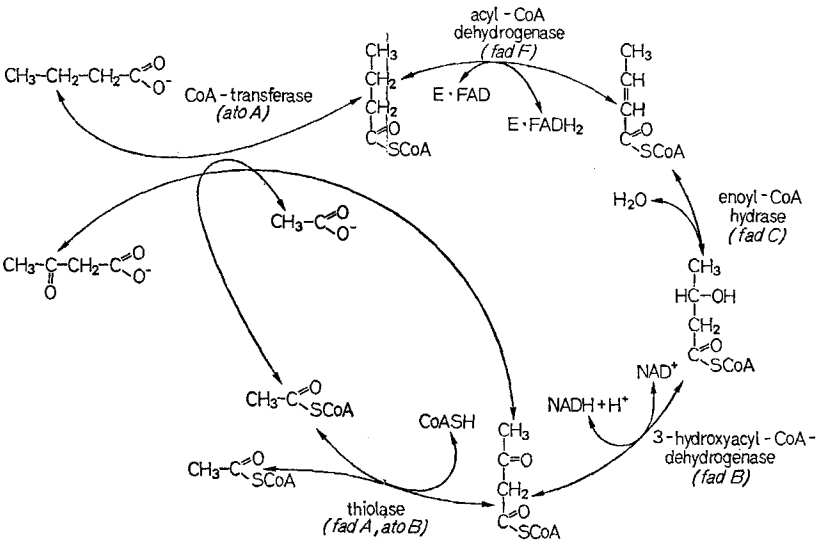


Fig. 1. *Pathways of butyrate and acetoacetate degradation in* E. coli

et al. [14]. In a further publication Vanderwinkel *et al.* [15] recently showed, in agreement with the results presented below, that the high thiolase activity and the CoA-transferase are both de-repressed by a one step mutation to the butyrate-positive phenotype. Whereas the occurrence of the CoA-transferase provided the mechanism for the activation of butyrate in these mutants, the second thiolase appeared superfluous, since the cells already contained such an activity. These observations can now be rationalized in the following way (Fig. 1): the combined action of the CoA-transferase, which also serves as an acetoacetyl-CoA : acetate CoA-transferase, and the second thiolase (thiolase II) enable the degradation of acetoacetate by the overall reaction:

Acetoacetate + CoASH → acetyl-CoA + acetate.

The structural genes for these enzymes form the *ato* operon (from *aceto*acetate) and their synthesis can be induced by acetoacetate. Since butyrate can neither de-repress the *fad* regulon (formerly called *old* regulon [4]) nor the *ato* operon, two mutations enabling constitutive expression of both operons are necessary for the degradation of butyrate.

MATERIAL AND METHODS

Reagents and Media

Butyryl-CoA was prepared from the anhydride [16], acetoacetate by saponification of the ester [17]. All other reagents and the preparation of media have been described previously [3—5]. When necessary, growth factors were added as follows: 20 μg/ml L-amino acids, 100 μg/ml thymine, 20 μg/ml other bases and 10 μg/ml vitamins. Streptomycin was used at a concentration of 200 μg/ml, nalidixic acid at 40 μg/ml (for *recA* strains only 10 μg/ml).

Bacteria and Bacteriophages

Table 1 lists the bacteria and bacteriophages used.

Bacteriological Methods

Auxotrophic markers were generally introduced by ultraviolet mutagenesis and penicillin selection. *fadR* and *atoC*^{c} mutants were selected as described before [4]. *ato*$^-$ mutants of strain *fadR16 fadA30 atoC*^{c}*49 his*$^-$ were obtained by plating a culture, mutagenized by nitrosoguanidine [18], on acetate plates. *ato*$^-$ mutants were found after replica-plating on butyrate plates. *recA* was introduced by the method of Low [19]. Curing of F-particles was performed with acridine orange at concentrations of 2.5—20 μg/ml, depending on the sensitivity of the strain to this reagent [20]. Conjugational crosses and transductions were performed as described previously [4].

Enzyme Assays

Uptake of fatty acids and enzyme activities were measured as before [3—5]. The CoA-transferase was determined with butyryl-CoA or acetoacetyl-CoA as substrate.

Butyryl-CoA : acetate CoA transferase [21] was estimated in an assay mixture containing 100 μmol Tris-HCl buffer pH 8.2, 0.2 μmol butyryl-CoA,

Table 1. *Bacterial strains and bacteriophages*

his, thr, leu, thi, trp, thy refer to the inability to synthesize histidine, threonine, leucine, thiamine, tryptophane, and thymine, respectively. *dsd* refers to the structural gene of D-serine deaminase. *strA*^{R} and *nalA*^{R} refer to resistance to streptomycin and nalidixic acid. *fad, ato, lac, gal, glp* refer to the ability to use oleate, acetoacetate, lactose, galactose or L-glycerol 1-phosphate as sole carbon source, respectively. For further details of nomenclature see Table 2. The chromosomal location of the genes is shown in Fig. 3

Strain	Characteristics	Sex	Origin
K12Ymel	prototrophic	F$^-$	U. Henning
fad-5, fadR16, fadR16 atoC^{c}1, fadR16 atoC^{c}1 fad-5	derivatives of K12Ymel		[4]
fadR16 fadA30	derivative of *fadR16*		this study
fadR16 fadA30 atoC^{c}49	*his*, derivative of *fadR16 fadA30*		this study
atoA28, atoB14, atoB58, atoB83, ato(AB)33, ato(AB)37	derivatives of *fadR16 fadA30 atoC^{c}49*		this study
fadR16 atoC^{c}49 B14	derivative of *fadR16*		this study
fadR16 fadD88 atoC^{c}1	*nalA*^{R}, derivative of *fadR16 fadD88*		this study
L106N	*strA*^{R}, *lacY, galK, thi, leu, thr, his, dsdA, thyA, nalA*^{R}, *glpT, trp*	F$^-$	H. J. Unsöld
H61	prototrophic, direction of transfer *thr-leu-lac* . . .	Hfr	P. Starlinger
H61 *fadR4*	derivative of H61		this study
H61 *fadR4 nalA*^{R} *glpT*	derivative of H61 *fadR4*		this study
H61 *fadR4 nalA*^{R} *glpT atoC^{c}1*	derivative of H61 *fadR4 nalA*^{R} *glpT*		this study
KL98	prototrophic, direction of transfer *aroC-purF-nalA* . . .	Hfr	B. Low
fadR16 fadA30 atoC^{c}49 recA strA^R/F′*ato*$^+$	chromosome: *his, nalA*^{R}	F′	this study
P1	P1kc		U. Henning
fr	male-specific RNA phage		W. Vielmetter

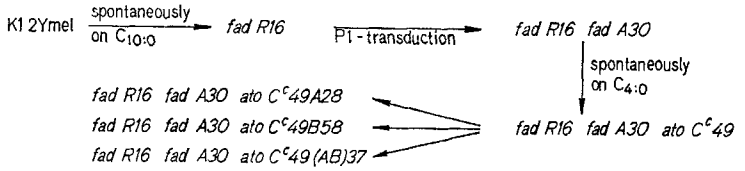


Fig. 2. *Derivation of* ato *mutants*

5 μg phosphotransacetylase (Boehringer Mannheim GmbH, Mannheim, Germany), 25 μmol potassium arsenate pH 8.2, 5 μmol potassium acetate pH 8.2; volume 1 ml; temperature 24 °C. The change in the absorbance at 232 nm ($\varepsilon = 4500$ cm^{-1} M^{-1}) was recorded after starting the reaction with potassium acetate.

Acetoacetyl-CoA : acetate CoA-transferase was estimated in an assay mixture containing 250 μmol Tris-HCl buffer pH 8.2, 2 μmol EDTA, 0.25 μmol NADH, 1 mg bovine serum albumin, 20 μg 3-hydroxyacyl-CoA dehydrogenase (Boehringer Mannheim GmbH, Mannheim, Germany), 0.2 μmol acetyl-CoA, 10 μmol sodium acetoacetate; volume 1 ml; temperature 24 °C. The change in absorbance at 366 nm was recorded after starting the reaction with sodium acetoacetate.

RESULTS

Isolation and Enzyme Levels of ato-*Mutants*

The nomenclature of the mutants used, their derivation and enzyme levels are summarized in Fig. 2 and Tables 2 and 3; their location on the *E. coli* linkage map is shown in Fig. 3. Among the various enzymes required for fatty acid breakdown, four of which are listed in Table 3, is an inducible thiolase, called thiolase I in this paper, which is absent in *fadA* mutants. The regulatory mutation from *fadR*$^+$ in the wild-type K12Ymel to *fadR16* leads to constitutive synthesis of these enzymes (Table 3). This mutation is a prerequisite for growth on butyrate, because $C_{4:0}$ cannot serve as inducer for the enzymes of fatty acid degradation [4]. The mutation which leads to growth on butyrate de-

Table 2. *Nomenclature and characteristics of mutants*

Genetic designation	Growth characteristics	Enzymatic and regulatory properties
Fatty acid degradation:		
fad$^+$	$> C_{12:0}$	wild type, inducible by fatty acids, formerly called *old*$^+$ [4]
fad$^-$	inability to grow on oleate	
fadA		lacks thiolase I
fadD		lacks acyl-CoA synthetase
fadR$^-$	$> C_{8:0}$	constitutive, formerly called *dec*$^+$, wild-type allele *fadR*$^+$
Acetoacetate degradation:		
ato$^+$	growth on acetoacetate	wild type, inducible by acetoacetate but not by butyrate
ato$^-$	inability to grow on acetoacetate or inability to grow on butyrate in combination with *fadR*$^-$ *atoC*^{c}	
atoA		lacks CoA-transferase
atoB		lacks thiolase II
ato(AB)		lacks CoA-transferase and thiolase II
atoC^c	growth on acetoacetate or, in combination with *fadR*$^-$, on butyrate	constitutive, formerly called *but-1*, wild-type allele *atoC*$^+$

Table 3. *Enzyme levels in* ato *mutants*

(A) The absolute specific activities for strain K12Ymel grown on acetate + oleate in $\mu mol \times min^{-1} \times mg\ protein^{-1}$: enoyl-CoA hydrase, 1.1; 3-hydroxyacyl-CoA dehydrogenase, 1.2; acyl-CoA synthetase, 0.0045; thiolase I, 0.082. (B) The specific activities for the *ato* mutants were determined in cells grown on acetate or acetate + oleate. Since the enzyme levels in both conditions were essentially the same, only average values are given. K12Ymel was grown in acetate + oleate. The absolute specific activities for strain *fadR16 fadA30 atoC*^{c}*49* in $\mu mol \times min^{-1} \times mg\ protein^{-1}$: enoyl-CoA hydrase, 2.5; 3-hydroxyacyl-CoA dehydrogenase, 5.2; thiolase II, 4.0; CoA-transferase, 0.6. The total amount of thiolase in the mutant *fadR16 fadA30* gives a lower limit for the residual synthesis of thiolase I and/or the basal rate of thiolase II synthesis. The values in brackets are therefore calculated by assuming that the rate of synthesis of thiolase I and/or thiolase II is $\leq 1\%$ of thiolase I activity in the induced wild-type K12Ymel. n.d. = not determined

Strain	Medium	Relative specific activity					
		Enoyl-CoA hydrase	3-Hydroxy-acyl-CoA dehydrogenase	Acyl-CoA synthetase	Thiolase I	Thiolase II	CoA-transferase
		%	%	%	%	%	%
A.							
K12Ymel	acetate + oleate	100	100	100	100		
	acetate	14	15	36	10		
fadR16	acetate + oleate	164	190	129	174		
	acetate	164	181	114	178		
fadR16 fadA30	acetate + oleate	59	86	161	1		
	acetate	56	87	166	1		
B.							
fadR16 fadA30 atoC^c*49*		100	100	n.d.	($\leq$ 0.05)	100	100
K12Ymel		84	52	n.d.	5	($\leq$ 0.05)	$<$ 2
fadR16 fadA30 atoC^c*49A28*		48	62	n.d.	($\leq$ 0.05)	50	$<$ 2
fadR16 fadA30 atoC^c*49B58*		120	115	n.d.	($\leq$ 0.05)	10	85
fadR16 fadA30 atoC^c*49(AB)37*		64	76	n.d.	($\leq$ 0.05)	0.2	$<$ 2

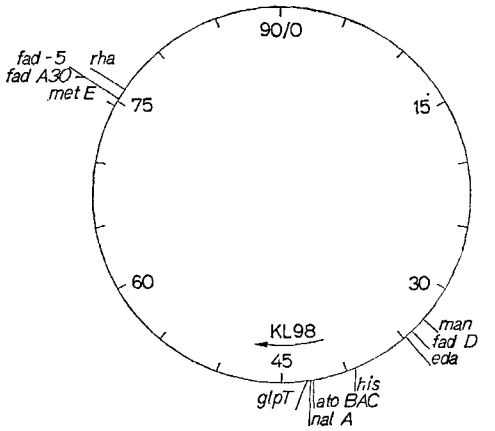


Fig. 3. *Positions of* fad *and* ato *mutations on the* E. coli *chromosome.* The map is drawn according to Taylor [22]. The arrow indicates the origin and direction of transfer of HfrKL98. *fadD88* has recently been shown to be co-transducible with *eda*, a gene involved in glucuronate degradation [23] (and D. Fraenkel, personal communication)

represses the synthesis of thiolase II which should be able to replace thiolase I. Therefore, the double mutant *fadR16 fadA30* was first constructed [4] which contains less than 1% of the thiolase activity of the parent *fadR16* and has a *fad*$^-$ phenotype (Table 3). It is not clear if this remaining activity is due to residual thiolase I activity and/or the basal rate of thiolase II synthesis. *fadR16 fadA30* gives rise to spontaneous mutants that grow on butyrate. This mutation is called *atoC*^{c}, because it de-represses the enzymes of acetoacetate breakdown. A typical example is the mutant *fadR16 fadA30 atoC*^{c}*49* (Fig. 2) which grows on butyrate, but, like the parent, is unable to use long-chain-length fatty acids as sole carbon source. By suitable back-crosses using the *metE* marker of strain PA 374 [4] it was verified that *fadR16 fadA30 atoC*^{c}*49* still contains the *fadA30* mutation. It is therefore clear that thiolase II can replace thiolase I for butyrate degradation *in vivo*.

Table 3 shows that in strain *fadR16 fadA30 atoC*^{c}*49* the specific activity of thiolase II is 20 times higher than the activity of thiolase I in the induced wild type. The factor for de-repression of thiolase II in strain *fadR16 fadA30 atoC*^{c}*49* is at least 2000. The same mutation leads to the synthesis of the butyryl-CoA : acetate CoA-transferase [14, 15], an enzyme which cannot be detected in the wild type with the relatively insensitive assay used. When coupled with phosphotransacetylase [21], the CoA-transferase activity is dependent on the addition of acetate and partially dependent on arsenate. It shows a pH optimum at 8.3 in Tris-HCl buffer and an apparent K_m of 60 μM for butyryl-CoA as substrate.

From strain *fadR16 fadA30 atoC*^{c}*49* butyrate-negative mutants can be isolated (Fig. 2). 26 from a total of 120 mutants tested (22%) have reduced levels of thiolase II and/or CoA-transferase. Since, as was shown in the final stage of this work, these mutants have lost at the same time the ability to grow on acetoacetate, they are called *ato*$^-$. The remaining butyrate-negative mutants presumably lack other enzymes required for butyrate breakdown (Fig. 1). The majority of the *ato* mutants (50%) has low levels of both enzymes, *i.e.* are *ato(AB), e.g. fadR16 fadA30 atoC*^{c}*49(AB)37*. The rest contain either no CoA-transferase and normal or somewhat reduced levels of thiolase II, *i.e.* are *atoA, e.g. fadR16 fadA30 atoC*^{c}*49A28*, or they have lost 90% of thiolase II while retaining the ability to form the CoA-transferase, *i.e.* are *atoB, e.g. fadR16 fadA30 atoC*^{c}*49B58*. The joint appearance of the two enzymes by the *atoC*^{c} mutation and their joint disappearance by the *ato(AB)* mutation suggests that the respective structural genes may form an operon.

Mapping of the ato *Locus*

The following experiments show that the three *ato* genes map closely together forming the sequence *atoCAB* between the markers *his* and *nalA* on the *E. coli* map (Fig. 3).

a) Mating of Hfr KL98 with several strains of the genotype *fadR16 fadA30 atoC*^{c}*49ato*$^-$ gives rise to butyrate-positive recombinants.

b) *atoC* as well as *atoA* and *atoB* are co-transducible with the *nalA* marker at a frequency of about 50%. This suggests that all three loci map closely together near the *nalA* gene.

c) Table 4 lists three-factor crosses of the *ato* locus with the neighbouring *nalA* and *glpT* markers. The first two transductions show that a high percentage of the selected *nalA* transductants receive both the *glpT* and the *atoC*^{c} traits. In transduction No. 3 *ato* segregates preferentially with *nalA* while *glpT* (No. 4) segregates preferentially with *nalA*, suggesting the order *glpT* — *nalA* — *ato*.

d) The close linkage to the *nalA* marker enabled reciprocal three-factor crosses, part of which are reported in Table 5. Both *atoA* and *atoB* map between *nalA* and *atoC* (No. 1 and 2). Since *atoB* maps between *nalA* and *atoA* (No. 3), the order is *nalA . . . atoBAC*. The *ato(AB)* mutations so far investigated, *(AB)33* and *(AB)37* (No. 4 and 5), presumably map between *atoA* and *atoC*. Since *atoC*^{c}*(AB)37* exerts a *trans*-dominant effect on *atoC*$^+$ (see below), the *ato(AB)* mutations are not reversions to *atoC*$^+$. More experiments are required to distinguish between two possibilities: either these mutations in fact map in the *A* gene and exert polarity on the expression of the *B* gene, an effect well known in other systems, or these mutations occur in a so far unspecified operator-promotor region between the *C* and *A* genes.

Table 4. *Order of* glpT, nalA *and* ato *loci*
The *ato*-phenotype was always scored on butyrate as sole carbon source. The derivative of H61 was obtained from the spontaneous mutant H61 *fadR4* by P1-transduction with strain L106N (selection for *nalA*^{R}) and *fadR16 atoC*^{c}*1* (selection on butyrate) as donors

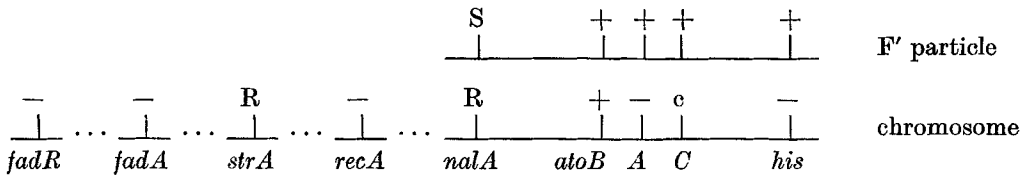
Transduction					
	P1 donor: H61 *fadR4 nalA*^{R} *glpT*$^-$ *atoC*^{c}*1*		*glpT*	*nalA*^{R}	*atoC*^{c}
	Recipient: *fadR16 fadA30 atoC*^{c}*49A28*		+	*nalA*^{S}	*ato*$^-$
1	Selected transductants	No. scored	Percentage of transductants that score as:		
	nalA^R	345	*glpT*$^-$	*atoC*^{c}	*glpT*$^-$ *atoC*^{c}
			69	57	34
	P1 donor: H61 *fadR4 nalA*^{R} *glpT*$^-$ *atoC*^{c}*1*		*glpT*	*nalA*^{R}	*atoC*^{c}*1*
	Recipient: *fadR16*		+	*nalA*^{S}	*ato*$^+$
2	Selected transductants	No. scored	Percentage of transductants that score as:		
	nalA^R	345	*glpT*$^-$	*atoC*^{c}	*glpT*$^-$ *atoC*^{c}
			71	59	41
	P1 donor: H61 *fadR4 nalA*^{R} *glpT*$^-$ *atoC*^{c}*1*		*glpT*	*nalA*^{R}	*atoC*^{c}
	Recipient: *fadR16 fadA30 atoC*^{c}*49A28*		+	*nalA*^{S}	*ato*$^-$
3	Selected transductants	No. scored	Percentage of transductants that score as:		
	atoC^c	276	*glpT*$^-$	*nalA*^{R}	*glpT*$^-$ *nalA*^{R}
			37	50	33
	P1 donor: *fadR16 atoC*^{c}*1*		+	*nalA*^{S}	*atoC*^{c}
	Recipient: H61 *fadR4 nalA*^{R} *glpT*$^-$		*glpT*	*nalA*^{R}	*atoC*$^+$
4	Selected transductants	No. scored	Percentage of transductants that score as:		
	glpT$^+$	670	*nalA*^{S}	*atoC*^{c}	*nalA*^{S} *atoC*^{c}
			61	31	25

Table 5. *Order of the* ato *loci*
All P1 donors have the *fadR*$^-$ *nalA*^{R} *glpT*$^-$ genotype. All recipients are *fadR*$^-$ *nalA*^{S}. All *atoC*^{c} strains are moreover *fadA30 his*$^-$. *atoC*^{c} recombinants were scored on butyrate as sole carbon source. The unselected marker *glpT* is inherited in reciprocal crosses at the same frequency

Transduction	Donor	Recipient	No. of *nalA*^{R} transductants scored	*atoC*^{c} recombinants	Order deduced
				%	
1	*atoC*^{c}*49A28*	*atoC*$^+$	1903	1.1	*nalA atoA atoC*
	atoC$^+$	*atoC*^{c}*49A28*	2443	8.7	
2	*atoC*^{c}*49B83*	*atoC*$^+$	1774	0.2	*nalA atoB atoC*
	atoC$^+$	*atoC*^{c}*49B83*	2681	8.5	
3	*atoC*^{c}*49B58*	*atoC*^{c}*49A28*	4156	0.7	*nalA atoB atoA*
	atoC^c*49A28*	*atoC*^{c}*49B58*	3252	2.3	
4	*atoC*^{c}*49A28*	*atoC*^{c}*49(AB)33*	1062	0.8	*nalA atoA ato(AB)*
	atoC^c*49(AB)33*	*atoC*^{c}*49A28*	1361	4.1	
5	*atoC*^{c}*49A28*	*atoC*^{c}*49(AB)37*	1790	0.5	*nalA atoA ato(AB)*
	atoC^c*49(AB)37*	*atoC*^{c}*49A28*	1791	7.6	
6	*atoC*^{c}*49B58*	*atoC*^{c}*49(AB)33*	2100	1.0	*nalA atoB ato(AB)*
	atoC^c*49(AB)33*	*atoC*^{c}*49B58*	2561	5.7	
7	*atoC*^{c}*49(AB)33*	*atoC*^{c}*49(AB)33*	1850	<0.1	
	atoC^c*49A28*	*atoC*^{c}*49A28*	1956	<0.1	
	atoC^c*49B58*	*atoC*^{c}*49B58*	1239	<0.1	

Dominance of atoC^c *over* atoC$^+$

The dominance relationship of the *atoC^c* and *atoC$^+$* alleles was tested in the following genetic situation:



A *recA* derivative [19] of strain *fadR16 fadA30 atoC^{c}49A28 strA^R nalA^{R}his$^-$* was crossed with strain KL98 selecting for heterozygotes on plates of glucose and mineral salts containing streptomycin. It was verified beforehand that KL98 behaves like K12Ymel, that is, it is *fadR$^+$ ato$^+$* and gives rise to *fadR$^-$ atoC^c* mutants as depicted in Fig. 2. The above heterozygote is sensitive towards the male-specific phage fr, can be cured by acridine orange and serves as an F′ donor. The latter property was used to transfer the F′ particle to various *fadR$^-$ ato$^-$ recA$^-$* strains. It can be seen from Table 6 that the presence of the *atoC^{c}49* allele in combination with neighbouring mutations in the structural genes for the *ato* enzymes exerts a *trans*-dominant effect on the *ato$^+$* alleles on the episome, enabling growth on butyrate. In the absence of an *atoC^c* allele no growth on butyrate is possible.

For unknown reasons the merodiploid state of the *ato* operon in the presence of the *fadR$^-$* mutation results in rather unfavourable growth properties of the cells. After growth in acetate or acetate plus oleate mineral salts medium part of the cells have a butyrate-negative phenotype. Since these cells are still *his$^+$*, we assume that under these conditions secondary mutants with lesions in *fad* genes are selected. Such apparent "reversions" are not obtained when the cells are grown on glucose as carbon source. Glucose represses the enzymes of the C-3 oxidation pathway even in constitutive *fadR$^-$* strains. Comparison of strain *atoC^{c}49* and *atoC^{c}49*/F′*ato$^+$* (Table 6, line 1) shows that the introduction of the episome does not increase the level of the *ato* enzymes. Since *atoC^c* appears to be *trans*-dominant over *atoC$^+$*, the absence of a gene-dosage effect may be caused by secondary interactions of the expression of the *ato* and *fad* operons. Since the inducer of the *ato* operon is now known (see below), the dominance of the *ato* operon can be studied in the future in a *fadR$^+$* background.

The merodiploid strains in lines 2—4 demonstrate that the *atoC^c* mutation on the chromosome with neighbouring mutations of the *atoA*, *atoB* or *ato(AB)* type enables the expression of CoA-transferase, thiolase II or both enzymes from the episomal *ato$^+$* operon. Thus, *atoC^{c}49* exerts a *trans*-dominant effect on the wild-type allele *atoC$^+$*

Table 6. *Dominance of* atoC^c *over* atoC$^+$

All strains listed under No. 1—4 carry the *fadR16* and *fadA30* mutations and are *recA*. The haploid strain listed under No. 5 is a *recA his$^-$* derivative of strain *fadR16*. The cells were grown with glucose as sole carbon source. The reference strain *atoC^{c}49* had the following specific activities: enoyl-CoA hydrase 0.08, 3-hydroxyacyl-CoA dehydrogenase 0.15, thiolase 0.81, butyryl-CoA : acetate CoA-transferase 0.18 $\mu mol \times min^{-1} \times mg$ protein^{-1}

	Strain	Growth on butyrate	Relative specific activity			
			Enoyl-CoA hydrase	3-Hydroxyacyl-CoA dehydrogenase	Thiolase II	CoA-transferase
			%	%	%	%
1	*atoC^{c}49*	+	100	100	100	100
	atoC^{c}49/F′*ato$^+$*	+	70	100	83	80
2	*atoC^{c}49A28*	−	120	150	81	< 4
	atoC^{c}49A28/F′*ato$^+$*	+	84	95	79	70
3	*atoC^{c}49B58*	−	93	125	3	42
	atoC^{c}49B58/F′*ato$^+$*	+	150	160	46	150
4	*atoC^{c}49(AB)37*	−	70	115	< 1	< 4
	atoC^{c}49(AB)37/F′*ato$^+$*	+	50	120	40	53
5	*ato$^+$*	−	300	380	< 1	< 4
	ato$^+$/F′*ato$^+$*	−	540	270	< 1	< 4

Table 7. *Induction of the* ato *enzymes by acetoacetate*
All strains were grown to an absorbance at 420 nm of 1.0—1.5 in acetate medium, washed once with buffer and then grown from an absorbance at 420 nm of 0.1 to 1—1.2 in the respective media indicated

Strain	Medium	Specific activity				
		Enoyl-CoA hydrase	3-Hydroxy-acyl-CoA dehydrogenase	Thiolase I + thiolase II	CoA-transferase	
					Butyryl-CoA: acetate	Acetoacetyl-CoA: acetate
		μmol × min^{-1} × mg protein^{-1}				
K12Ymel	acetate	0.16	0.32	0.018	< 0.01	< 0.006
	acetate + acetoacetate	0.12	0.15	2.0	0.17	0.39
	acetoacetate	0.11	0.18	2.5	0.15	0.39
fadR16 atoC^{c}1	acetate	4.0	4.8	6.5	0.38	0.94
	acetate + acetoacetate	1.9	2.8	4.9	0.8	1.5
	acetoacetate	1.9	2.2	7.5	0.86	2.1
fad-5	acetate	< 0.002	< 0.005	0.0008	< 0.01	< 0.006
	acetate + acetoacetate	< 0.002	< 0.005	2.9	0.34	1.0
	acetoacetate	< 0.002	< 0.005	3.5	0.4	1.1
fadR16 atoC^{c}1 fad-5	acetate	< 0.002	< 0.005	2.7	0.36	0.65
	acetate + acetoacetate	< 0.002	< 0.005	4.7	0.54	1.0
	acetoacetate	< 0.002	< 0.005	7.9	0.89	2.6

even in the presence of the pleiotropic (*AB*) mutation. While these experiments suggest some type of positive control effect of the *atoC^c* gene, firm conclusions can only be drawn after further analysis of many independently isolated *atoC^c* mutants.

Induction of the ato *Enzymes*

Although the high de-repression factor of the *atoC^c* mutation for the two enzymes of the *ato* operon and the *trans*-dominant effect of *atoC^c* over *atoC$^+$* appeared interesting enough to justify further genetic and biochemical analysis, the normal function of the CoA-transferase and of thiolase II remained obscure. A number of acids (butyrate, isobutyrate, DL-3-hydroxybutyrate, valerate, isovalerate) did not serve as inducers for the *ato* enzymes. It was found in the final stage of this study that Califano and Villano [24] and Lenti [25] had shown in 1947/48 that *E. coli* can respire with acetoacetate as substrate. As already pointed out in the Introduction (Fig. 1), acetoacetate is the only substrate which can be channelled into the acetate pool with the two *ato* enzymes.

The wild-type K12Ymel, *fadR$^-$* mutants (*e.g. fadR16*), mutants containing the *atoC^c* mutation (*e.g. fadR16 atoC^{c}1*) and the pleiotropic mutant *fad-5*, which lacks several enzymes of the C-3 oxidation pathway (Table 7), all grow on acetoacetate as sole carbon source. The butyrate-negative, but acetoacetate-positive phenotype of the mutant *fadR16 atoC^{c}1 fad-5* [4] demonstrates that the products of the *metE*-linked genes of the *fad* regulon are not required for acetoacetate breakdown. On the other hand, all *ato$^-$* mutants, *i.e. A*, *B* or (*AB*), described above are unable to grow on acetoacetate. Since *atoA* mutants (*e.g. fadR16 fadA30 atoC^{c}49A28*) lose the ability to grow on butyrate or acetoacetate at the same time, the CoA-transferase must be able to activate both acids.

Table 7 shows that acetoacetate serves as an inducer of the *ato* enzymes in the wild-type, whereas acetoacetate has no effect on the enzyme levels in the constitutive strain *fadR16 atoC^{c}1*. As expected, the CoA-transferase can use either butyryl-CoA or acetoacetyl-CoA as substrate. Of special interest is the strain *fad-5* since it contains a very low level of thiolase I. In accordance with the de-repression by the *atoC^c* mutation an induction factor of 3000—4000 is observed.

Uptake of Butyrate

It was originally suggested by Vanderwinkel *et al.* [14] that the CoA-transferase may be part of a butyrate transport system. In our hands this enzyme is soluble, *i.e.* it is found in the supernatant of an extract prepared by sonication. This behaviour is in contrast to the partial association of the fatty acyl-CoA synthetase with the membranous fraction, an enzyme apparently involved in fatty acid uptake [5]. It has been found by Vanderwinkel *et al.* and by Salanitro and Wegener [7, 8, 15] that only mutants which use butyrate as sole carbon source can take up this fatty acid. In agreement with these authors we have found the uptake of butyrate to be a saturable process ($K_m = 180$ μM) in *atoC^c* mutants (*e.g. fadR16 fadA30 atoC^{c}49*). Table 8 shows that the strain constitutive for the enzymes

Table 8. *Uptake of butyrate*
Uptake of butyrate (0.5 mM, spec. act. 0.2 μCi/μmol) or octanoate (0.3 mM, spec. act. 0.17 μCi/μmol) was followed over 10 min in cells growing aerobically on mineral salts medium + xylose. 100% refers to a rate of uptake of 10.3 μmol × min^{-1} × mg $protein^{-1}$ for butyrate and 3 μmol × min^{-1} × mg $protein^{-1}$ for octanoate uptake, respectively

Strain	Relative rate	
	butyrate	octanoate
	%	%
fadR16 fadA30 atoC^c49	100	100
fadR16 fadA30 atoC^c49B58	4.4	90
fadR16 fadA30 atoC^c49A28	< 1	80
fadR16 fadA30 atoC^c49(AB)33	< 1	80
fadR16	< 1	165
fadR16 atoC^c1	50	43
fadR16 atoC^c1 fad-5	< 1	30

of the C-3 oxidation pathway (*fadR16*) is unable to take up butyrate, whereas the *atoC^c* mutation causes the appearance of such an activity. Low levels of thiolase II (*atoB58*) drastically reduce the rate of uptake. In the absence of the CoA-transferase (*atoA28*) or in the presence of the pleiotropic *ato(AB)* mutation uptake of butyrate is completely suppressed. The last strain listed in Table 8 shows that in the presence of the pleiotropic *fad-5* mutation, which lacks several enzymes of butyrate breakdown (3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydrase), butyrate uptake is also impossible. It thus appears that, as in the transport systems for C_4-dicarboxylic acids [26] and long-chain-length fatty acids [5], the translocation step is intimately coupled to the further metabolism of the transported substrate. Table 8 also lists the rate of octanoate uptake which is part of the transport system for long and medium-chain-length fatty acids. All *ato* mutants show a somewhat reduced rate of octanoate uptake compared to the parent *fadR16*. In particular, *fadR16 atoC^c1* gives a reduction by a factor of four. This effect may be related to the reduced ability of *fadR^- atoC^c* mutants to grow on medium-chain-length fatty acids (see below and [4]).

Relationship between Butyrate and Long-Chain-Length Fatty-Acid Degradation

A number of additional observations which are described in detail elsewhere [27] should be briefly mentioned.

As has been shown before [4], *fadR16 atoC^c* mutants grow more slowly on long-chain-length fatty acids than the parent *fadR16*. The reason for this behaviour remains obscure although it is in accordance with the observed effect on octanoate uptake (Table 8). It is, however, possible to use this property for selection of "revertants" from *fadR16 atoC^c1* on dodecanoate as sole carbon source. These "revertants" are either of the *atoC^cA^-* or *(AB)^-* type or they are still *fadR16 atoC^c1* as shown by enzymatic assays. All these mutants show a growth behaviour like the original parent *fadR16* on long-chain-length fatty acids, while only the second type grows on butyrate. Thus, the inhibition of growth on long-chain-length fatty acids caused by the *atoC^c* mutation can either be relieved by a mutation in the CoA-transferase gene or by a secondary mutation of unknown nature.

Some interesting problems are raised by the presence of the two thiolases. As shown above, thiolase I is not required for butyrate breakdown when thiolase II is formed. However, thiolase II cannot replace thiolase I in the degradation of long-chain-length fatty acids because strain *fadR16 fadA30 atoC^c49* is still *fad^-*. Presumably, thiolase II has a narrower chain-length specificity than thiolase I. Thiolase I can also partially replace thiolase II in butyrate breakdown. This could be demonstrated by constructing the strain *fadR16 atoC^c49B14* which contains thiolase I and some residual thiolase II activity. This strain can slowly grow on butyrate in contrast to the parents *fadR16* and *fadR16 fadA30 atoC^c49B14*. In summary, the two thiolase activities can replace each other in butyrate degradation.

The mapping of the *ato* genes made it possible to cross the *atoC^c* mutation into the wild-type K12-Ymel. Although such a strain is constitutive for the *ato* enzymes, it cannot grow on butyrate because it is repressed for the enzymes of C-3 oxidation. According to our criteria for growth [4], effective butyrate degradation is only possible in the presence of the *fadR^-* mutation. Finally, the *atoC^c* mutation was crossed into strain *fadR16 fadD88* [5] which lacks the acyl-CoA synthetase. The resultant triple mutant *fadR16 fadD88 atoC^c1* forms thiolase II and CoA-transferase and synthesizes all enzymes of C-3 oxidation, save the acyl-CoA synthetase, constitutively. This strain grows as expected on butyrate but not on long-chain-length fatty acids. The acyl-CoA synthetase is therefore not involved in butyrate degradation.

Several butyrate-growing strains have been kindly supplied to us by Dr Vanderwinkel and Drs W. Kay and H. L. Kornberg, part of which have been selected from mutants synthesizing the enzymes of the glyoxylate cycle constitutively. In accordance with Vanderwinkel *et al.* [15] their strain D5H3D7 was found to be *fadR^+ ato^+* whereas their butyrate-positive mutant V10 is partially de-repressed for the enzymes of the C-3 oxidation pathway and forms thiolase II and CoA-transferase (genotype *fadR^- atoC^c*). The strains obtained from Drs Kay and Kornberg were essentially all *fadR^- atoC^c*. Our own *fadR^- atoC^c* mutants are inducible for malate synthase. It is therefore concluded

that constitutivity for the enzymes of the glyoxylate bypass is not required for the butyrate-positive phenotype.

DISCUSSION

An induction factor of at least 2000 to 3000 by acetoacetate places the *ato* operon in line with highly de-repressible operons like the *lac* operon [28]. It consists of two adjacent structural genes and a closely linked regulatory gene. The linkage of the structural genes is supported by the mapping data and the occurrence of the pleiotropic *ato(AB)* mutants. The high proportion of polar *ato(AB)* mutants among all *ato*$^-$ mutants is not withous precedent in other systems [29]. More experiment- are required in order to decide if the (*AB*) mutat tions are regulatory mutations in an operator-promotor region or structural gene mutations within the *A* gene. It may, furthermore, be expected that at least one additional gene product, a transport protein, is required for acetoacetate degradation.

The regulatory gene *atoC* has so far been studied in its inducible wild-type state and the constitutive *atoC*^{c} state. From the dominance behaviour of the *atoC*^{c}*49* mutation it appears that in the *C*^{c} state the *C* gene produces a diffusible product which leads to the expression of the genes of the episomal *ato* operon. A positive type of control may therefore be suggested. Two additional observations have to be emphasized. Firstly, *atoC*^{c} mutations occur with a rather high spontaneous rate of 10^{-5}—10^{-6}. Secondly, the de-repressed enzyme levels of different *atoC*^{c} mutants are essentially the same and no intermediate types have been observed. This behaviour may be the result of the selection method used for isolating *atoC*^{c} mutants.

The properties of the *ato* system can be compared with other operons. For example, the *trans*-dominant *i*$^{-d}$ mutants in the regulator gene of the lactose operon [30—33] exert their behaviour by negative complementation. They are, however, only a small group among constitutive *i*$^-$ mutants. Since the dominance of only one *atoC*^{c} mutant has been studied so far, there is a definite possibility that this mutation is similar to an *i*$^{-d}$ mutant. Further analysis of independently isolated *atoC*^{c} mutants may reveal that the majority of them are *trans*-recessive, supporting a negative model of control. A gradient of de-repressed enzyme levels has been shown for the *C*^{c} mutations in the arabinose operon. However, this type of mutation is recessive against *araC*$^+$ [34]. Only further experiments can decide if the *ato* system really has unique features or can be accommodated within the framework of current knowledge on regulation of gene expression.

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