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Growth of *Escherichia coli* on Short-Chain Fatty Acids: Nature of the Uptake System

JOSEPH P. SALANITRO[1] AND WARNER S. WEGENER

Department of Microbiology, School of Medicine, Indiana University, Indianapolis, Indiana 46202

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Mutants of *Escherichia coli* K-12 which grow on butyrate and valerate were studied with respect to uptake of these substrates. To utilize short-chain and medium-chain fatty acids, *E. coli* must synthesize the β-oxidation enzymes constitutively. In addition, growth on the C_4 and C_5 acids requires a second mutation which permits entry of these substrates. At *p*H 5, both in the parent and mutant strains, butyrate and valerate penetrate as the undissociated acids but appear not to be activated and thus inhibit growth. At *p*H 7, the parent strain is not permeable to the anions, whereas the mutant concentrates these substrates. There appear to be two components of the uptake system, a nonspecific diffusion component and an energy-linked activating enzyme. Two mutant types which take up short-chain fatty acids are described. One synthesizes the uptake system constitutively and is inhibited by 4-pentenoate when cultured on acetate. In the other, the uptake system is inducible, and the strain is pentenoate-resistant when grown on acetate but pentenoate-sensitive when cultured on butyrate or valerate.

Escherichia coli K-12, constitutive for the enzymes of the glyoxylate bypass, grows by using long-chain ($>C_{12}$ to C_{18}) but not medium-chain (C_6 to C_{11}) or short-chain (C_4 and C_5) fatty acids as the sole source of carbon. Mutants which utilize medium- and short-chain normal monocarboxylic acids were described in the accompanying publication (13). Derepression of the β-oxidation operon by long-chain fatty acids and the isolation of β-oxidation-constitutive mutants were described previously for other K-12 strains (9, 16).

In the strain employed here (13), fatty acids having 12 or more carbons caused derepression of synthesis of the β-oxidation enzymes. β-oxidation-constitutive mutants grew using medium- but not short-chain fatty acids as the sole source of carbon. Activation systems for long- and medium-chain but not short-chain fatty acids appear to be regulated coordinately with the β-oxidation enzymes. Growth data suggest physiologically distinct activation systems for long-chain and medium-chain acids (13). Overath et al. (9, 10) described an adenosine triphosphate (ATP)-dependent fatty acid acyl-coenzyme A (CoA) synthetase from *E. coli* K-12 which activates long-chain acids but is inactive with substrates less than C_8. In a different K-12 strain, Samuel et al. (14, 15) demonstrated two synthetase activities, one for long-chain acids and another exhibiting maximum activity with C_6.

A second mutation is required for β-oxidation-constitutive mutants to utilize butyrate and valerate (13). Growth of short-chain fatty acid mutants (type N_3) on acetate is inhibited by 4-pentenoate. It is proposed that this unsaturated C_5 fatty acid is activated as the acyl-CoA ester but is not metabolized and hence causes depletion of free CoA·SH. This growth inhibition can be relieved by addition of CoA precursors to the culture medium. 4-Pentenoate also has been shown to inhibit fatty acid oxidation in pigeon liver homogenates (4). In the latter system, 4-pentenoyl-CoA, acrylyl-CoA, and the corresponding carnitine esters accumulate. 4-Pentenoate inhibition of fatty acid oxidation in these homogenates can be reversed both by CoA and (−) carnitine.

In contrast to N_3, growth of the parent strain is not inhibited by 4-pentenoate. Pentenoate-resistant revertants of N_3 (type N_3PR) lose the ability to grow at the expense of short-chain but not medium-chain fatty acids. These data suggest that the mutation in N_3 which permits growth on butyrate and valerate is one which regulates uptake of these acids. The present communication describes characteristics of the uptake system.

MATERIALS AND METHODS

Growth conditions and preparation of cells. The

[1] Present address: Biological Sciences, Research Center, Shell Development Co., Modesto, Calif. 95352.

strains employed and the commercial sources of the fatty acids and *l*-^{14}C-labeled substrates were described in the preceding report (13). Cells were grown at 37 C in a mineral salts medium using fatty acids as the sole source of carbon as previously described (13).

For uptake studies, cells were grown at 37 C in mineral salts, containing the carbon source indicated, to the mid to late exponential phase, centrifuged at room temperature, washed with mineral salts (*p*H 7.0), and resuspended in same to a concentration of 20 mg of wet cells per ml. For preparation of shocked cells, cell suspensions were subjected to the cold osmotic shock precedure described by Neu and Heppel (8). Lysozyme-treated cells and lysozyme-ethylenediaminetetraacetic acid (EDTA) spheroplasts were prepared in 0.5 M sucrose according to the procedure of Birdsell and Cota-Robles (2).

Uptake studies. Uptake of fatty acids was measured by incubating washed cell suspensions in the presence of *l*-^{14}C substrates for varying periods of time and then determining the amount of radioactivity taken up. Rates of uptake are expressed as nmoles of substrate taken up in 1 min per mg of wet cells. The incubation mixture contained, in a final volume of 0.5 ml, 2 mg of wet cells, 0.5 μmole (0.5 μCi) of *l*-^{14}C fatty acid, and mineral salts. Unlabeled substrates and inhibitors were added to incubation mixture where indicated. Cells were preincubated at 37 C for 10 min with shaking in a gyratory water bath, and then the sodium salt of the *l*-^{14}C fatty acid was added. At appropriate intervals, 0.1-ml samples of the cell suspension were withdrawn and pipetted on to 0.45-μm Millipore filter discs. The filters were washed with 10 ml of ice-chilled buffer [0.15 M NaCl and 0.5 M $MgCl_2$ in 10 mM tris(hydroxymethyl)-aminomethane-hydrochloride, *p*H 7] and dried with a heat lamp. For determination of radioactivity, the filters were placed in 10 ml of scintillation fluid containing 6 g of 2,5-diphenyloxazole and 300 mg of 1,4,-bis-2-(5-phenyloxazolyl)benzene (Packard Instrument Co.) in 600 ml of toluene and 300 ml of absolute ethanol. The radioactivity was then counted in a Packard liquid scintillation spectrometer.

RESULTS

In the preceding publication (13), β-oxidation-constitutive mutants which grow using medium- and short-chain fatty acids as the sole source of carbon were described. The growth characteristics of these mutants are summarized in Table 1. Both N_3 and D_1 synthesize the β-oxidation enzymes constitutively and grow on medium-chain acids. N_3 but not D_1 grows at the expense of short-chain acids, and, in the former, growth on acetate is inhibited by addition of 4-pentenoate to the medium. Pentenoate-resistant revertants (N_3PR) derived from N_3 lose the ability to grow at the expense of short-chain but not medium-chain acids.

After extended incubation of D_1 in minimal media containing butyrate or valerate, mutants which grew on the C_4 or on the C_5 acid, respectively, were selected. When transferred from succinate, D_1B grows on butyrate and, after an extended lag, grows at the expense of valerate. D_1V grows on valerate but not on butyrate. These mutants differ from N_3 in that they are pentenoate-resistant when cultured on acetate but pentenoate-sensitive when cultured on the C_4 or C_5 short-chain acids. This behavior appears to reflect the fact that the short-chain fatty acid uptake system is constitutive in N_3 but inducible in D_1B and D_1V.

Characteristics of the short-chain fatty acid uptake system. The finding that growth on butyrate and valerate was correlated with sensitivity to growth inhibition by 4-pentenoate suggested that N_3 differed from the parent and the D_1 mutant with respect to ability to uptake short-chain fatty acids. Table 2 compares these strains with respect to uptake of butyrate and valerate. For these assays, cells were grown on acetate and suspended in mineral salts. The resting-cell suspensions were then incubated in mineral salts with or without addition of glucose, and uptake of the *l*-^{14}C fatty acids was measured after 1, 5 and 30 min of incubation. N_3 exhibits significant uptake of butyrate and valerate, whereas the parent strain does not. The amount of ^{14}C-butyrate taken up by the parent strain is comparable to that shown by N_3 incubated in the presence of 5 mM azide. This basal level of fatty acid uptake appears to reflect nonspecific adsorption and increases with an increase in fatty acid chain length. N_3PR and D_1 and also the D_1B and D_1V mutants (not shown) exhibit only a low butyrate uptake over a period of 30 min. In these mutants, as well as in the parent, the basal rate of uptake of valerate is greater than that of butyrate and is stimulated somewhat by preincubation of cells in the presence of an energy source.

Incorporation of short-chain fatty acids by resting-cell suspensions of N_3 is enhanced by preincubation with an energy source and is blocked by azide. Addition of chloramphenicol or of detergents such as Brij-35 or Triton X-100 to the incubation mixture does not alter uptake of these substrates by the parent or mutants. Short-chain fatty acid uptake showed saturation kinetics with apparent K_m values of 0.08 mM for butyrate and 0.4 mM for valerate.

The effect of cold osmotic shock on the ability of N_3 to uptake butyrate is shown in Table 3 (experiment A). In this experiment, cells were suspended in buffer containing sucrose plus EDTA, centrifuged, and rapidly resuspended in cold buffer containing Mg^{2+} as described by Neu and Heppel (8). Such shocked cells showed a marked reduction in uptake of butyrate. Preincubation of shocked cells for 10 min in the pres-

TABLE 1. *Summary of growth behavior of the parent and mutant cell types on fatty acids*[a]

Fatty acid	Parent	N_3	N_3PR	D_1	D_1B	D_1V
Butyrate	−	+	−	−	+	−
Valerate	−	+	−	−	−[b]	+
C_6-C_{11}	−	+	+	+	+	+
4-Pentenoate	O	(I)	O	O	O[c]	O[c]

[a] With the exception of 4-pentenoate, the fatty acids indicated were used as the sole source of carbon as previously described (13). The parent and mutant strains are similar with respect to growth on acetate, propionate, and oleate. Symbols: −, no growth in 120 hr; +, grows with no significant lag when transferred from succinate; O, 4-pentenoate (20 mM) neither supports growth nor inhibits growth on acetate; (I), 4-pentenoate (5 mM) inhibits growth on acetate.

[b] D_1B grows on valerate but only after a long lag.

[c] 4-Pentenoate (20 mM) does not inhibit growth on acetate but does inhibit growth at the expense of the C_4 or C_5 fatty acids.

TABLE 2. *Comparison of uptake of short-chain fatty acids in parent and mutant strains*[a]

Incubation conditions	Pulse (min)	Uptake[b] by			
		Parent	N_3	N_3PR	D_1
Buffer (^{14}C-butyrate)	1	.15	0.61[c]	.13	.14
	5	.16	4.5	.18	.18
	30	.15	16.6	.25	.21
Glucose (^{14}C-butyrate)	1	.15	1.6	.26	.17
	5	.15	8.7	.31	.25
	30	.24	61.2	.38	.37
Buffer (^{14}C-valerate)	1	.24	0.56	.17	.21
	5	.27	3.5	.26	.27
	30	.31	15.3	.34	.29
Glucose (^{14}C-valerate)	1	.29	2.0	.24	.21
	5	.32	10.5	.47	.65
	30	.48	63.3	.76	.92

[a] The parent and mutant strains were grown on acetate and suspended in mineral salts (*p*H 7) as described. Cell suspensions were preincubated for 10 min at 37 C with or without 5 mM glucose and incubated in the presence of 1 mM butyrate-*1*-^{14}C or valerate-*1*-^{14}C. Radioactivity taken up in 1, 5, and 30 min was determined as described.

[b] Expressed as nanomoles of fatty acid taken up in the time period indicated per milligram of wet cells.

[c] The amount of butyrate and valerate taken up in a 5-min period by N_3 preincubated in the presence of 5 mM azide was 0.13 and 0.18 nmoles per mg of wet cells, respectively.

ence of CoA or ATP did not restore uptake. Likewise, preincubation with glucose or with acetate had little effect on uptake. Preincubation of shocked cells with succinate and to a lesser degree with pyruvate partially restored uptake of butyrate. These data suggest that the shock procedure causes cells to be depleted of energy and that in such cells succinate but not glucose acts to provide energy required for butyrate uptake.

E. coli has been shown to possess binding proteins which are involved in the transport of various substances. Binding proteins for sulfate (11), phosphate (7), amino acids (1, 12, 18), galactose (3), and arabinose (5) are released from the cell by cold osmotic shock treatment. Shocked cells show reduced uptake of these substrates. In general, uptake can be partially restored by addition of shock fluid or purified binding protein to shocked cells. In the experiments described here, incubation of shocked cells with either freshly prepared or concentrated shock fluid did not restore butyrate uptake. Likewise, addition of shock fluid to suspensions of whole cells of the parent strain did not result in uptake activity. A number of attempts were made to demonstrate short-chain fatty acid binding activity in cell-free shock filtrates prepared from N_3. For binding experiments, the equilibrium dialysis procedure was employed, and binding activity for leucine was assayed as a positive control. While ^{14}C-leucine binding activity was readily apparent, no binding activity could be demonstrated for ^{14}C-

TABLE 3. *Uptake of butyrate by cold osmotic-shocked and spheroplast preparations of* N_3[a]

Cell treatment	Preincubation addition[b]	Uptake activity (%)[c]
Expt A		
Control cells	None	100
Control cells	Glucose	240
Shocked cells	None	20
Shocked cells	ATP	18
Shocked cells	CoA	30
Shocked cells	Glucose	31
Shocked cells	Acetate	25
Shocked cells	Pyruvate	48
Shocked cells	Succinate	72
Shocked cells	Shock fluid	37
Expt B		
Control cells	0.5 M Sucrose	100
Lysozyme	0.5 M Sucrose	85
Lysozyme-EDTA	0.5 M Sucrose	15

[a] N_3 was cultured in acetate media and resting-cell suspensions were subjected to the cold osmotic shock procedure or to lysozyme or lysozyme-ethylenediaminetriacetate (EDTA) treatment as described.

[b] Cells were preincubated for 10 min at 37 C in mineral salts containing the additions indicated. Glucose, acetate, pyruvate, and succinate were added at 5 mM and CoA and adenosine triphosphate (ATP) at 1 mM.

[c] Butyrate taken up in 1 min by control cells incubated in mineral salts was given a value of 100, and all other activities are expressed as relative per cent.

butyrate or -valerate with concentrations of 10^{-3} to 10^{-6} M. The lack of a demonstrable binding protein for short-chain fatty acids is in agreement with the low affinity of these substrates for the uptake system.

Experiments designed to assess the effect of removal of cell wall material on butyrate uptake are shown in Table 3 (experiment B). In this experiment, cells were suspended in sucrose and treated with lysozyme or lysozyme plus EDTA as described by Birdsell and Cota-Robles (2). Although cells treated with lysozyme in the absence of EDTA retained most of the activity of whole cells, spheroplasts prepared using lysozyme plus EDTA showed a marked decrease in butyrate uptake. Uptake of ^{14}C in the cells was assayed by using mineral salts containing 0.5 M sucrose. The effect of exposure of cells to EDTA in the absence of sucrose or lysozyme was not determined.

Effect of related short-chain acids on uptake of butyrate. The effect of unlabeled short-chain acids on the uptake of ^{14}C-butyrate and -valerate by N_3 is shown in Table 4. In this study, cells were incubated in the presence of the unlabeled fatty acid indicated for 1 min and then pulsed for 1 min with the ^{14}C substrate. As expected, the addition of unlabeled butyrate competitively inhibited ^{14}C-butyrate uptake. Similarly, preincubation with unlabeled valerate inhibited uptake of ^{14}C-valerate. Although unlabeled butyrate markedly inhibited uptake of ^{14}C-valerate, the inhibition of ^{14}C-butyrate uptake by unlabeled valerate was much less pronounced. These data suggest that butyrate and valerate are concentrated by an uptake system which has at least one component in common. The fact that valerate is less effective than is butyrate as a competitive inhibitor of ^{14}C-butyrate uptake reflects the fact that the uptake system exhibits less affinity for the C_5 than for the C_4 substrate and is in accord with the relative affinities determined for these substrates.

TABLE 4. *Effect of related fatty acids on uptake of butyrate and valerate*[a]

Unlabeled substrate	^{14}C-substrate	Inhibition of uptake (%)[b]
Expt A		
None	Butyrate	0
Butyrate	Butyrate	82
Valerate	Butyrate	26
None	Valerate	0
Butyrate	Valerate	77
Valerate	Valerate	80
Expt B		
None	Butyrate	0
Acetate	Butyrate	64
4-Pentenoate	Butyrate	81
Hexanoate	Butyrate	8
2-Butenoate	Butryate	38
3-Butenoate	Butyrate	89
2-Methylpropionate	Butyrate	0
2-Methylbutyrate	Butyrate	0
2-Hydroxybutyrate	Butyrate	0
2-Aminobutyrate	Butyrate	0

[a] N_3 was grown on acetate, and resting-cell suspensions were prepared as described. Cells were preincubated at 37 C for 9 min in mineral salts and the unlabeled substrates (5 mM) were added. After 1 min, the ^{14}C-fatty acid (1 mM) was added and radioactivity taken up in 1 min was determined.

[b] ^{14}C taken up in the absence of added unlabeled fatty acid was employed as the control.

The effect of related short-chain acids on butyrate uptake is shown in Table 4 (experiment B). It is of interest that acetate, which would be expected to enhance butyrate uptake by providing energy, inhibits butyrate uptake. This inhibition may be explained on the basis that acetate is activated as the acyl-CoA ester and thus reduces the amount of free CoA·SH which is available for the activation of butyrate. 4-Pentenoate, as would be expected from the growth data, is an effective inhibitor of butyrate uptake. This acid is not metabolized and may inhibit uptake of butyrate both by competing for a component of the uptake system and by tying up CoA·SH as an acyl-CoA ester. The C_6 saturated acid does not significantly inhibit butyrate uptake. Uptake of hexanoate appears to be effected by a medium-chain activation system which is coordinately regulated with the β-oxidation operon (13). 2-Butenoate and 3-butenoate inhibit butyrate uptake by 38 and 89%, respectively. The difference in degree of inhibition probably reflects differences in the affinity of the uptake system for these substrates. Both of these unsaturated C_4 acids support growth of N_3 but are not utilized by the parent or D_1 or N_3PR mutants (13). The α-methyl-substituted C_4 and C_5 acids do not alter uptake of butyrate and neither support nor inhibit growth in N_3. Studies with ^{14}C-methylpropionate indicate that this substrate is not taken up by the parent or mutants. Likewise, 2-hydroxy- and 2-amino-butyrate do not alter ^{14}C-butyrate uptake. These substrates do not support but inhibit growth on acetate in both N_3 and the parent strain. These compounds appear to enter by a different uptake system and are probably not activated as acyl-CoA esters. The 3- and 4-hydroxy- and 4-amino-C_4 acid derivatives (not shown) neither inhibit butyrate uptake nor support or inhibit growth of the parent or mutants.

Effect of pH on uptake of short-chain fatty

acids. The data shown in Fig. 1 indicate that entry of short-chain fatty acids is markedly influenced by the *p*H of the medium. For these studies, tryptone broth with or without addition of 4-pentenoate was buffered using Sorensen's phosphate over a *p*H range of 5 to 8 and sterilized by filtration. Growth rates of the parent and N_3 mutant in the absence of pentenoate were similar over this range of *p*H. In the parent strain, growth on tryptone was inhibited by pentenoate at *p*H values of 6 and below, but growth was not altered by pentenoate at *p*H values of 6.5 and above. In contrast, 4-pentenoate inhibited growth of N_3 at all *p*H values tested. A similar *p*H-dependent inhibition pattern was exhibited by the parent when butyrate or valerate was substituted for pentenoate. This *p*H effect also was evident when acetate-mineral salts was substituted for tryptone.

Table 5 compares the uptake of butyrate and valerate in the parent and N_3 mutant incubated in buffer at *p*H 5 and at *p*H 7 in the presence and absence of glucose. The pK_a values for both butyrate and valerate are 4.7 to 4.8. At *p*H 5, the parent is permeable to these acids, but entry is not stimulated by glucose. Under these conditions, the ^{14}C substrates are not incorporated into trichloroacetic acid-insoluble material and appear not to be activated as acyl-CoA esters. At *p*H 5, N_3 is similar to the parent with respect to entry of these acids. As noted previously, the parent is not permeable to short-chain fatty acids at *p*H 7. As the result of a mutation, N_3 appears to have gained the ability both to effect permeation of short-chain acids at *p*H 7 and to activate these substrates as acyl-CoA esters. In addition, N_3 is constitutive for the enzymes of β-oxidation (13) and thus can grow at the expense of these substrates.

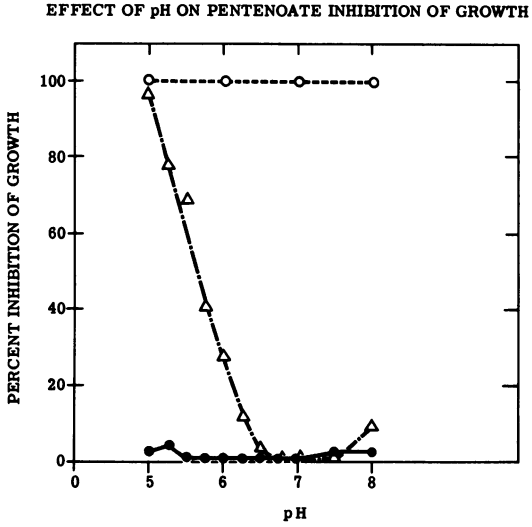

FIG. 1. *Effect of pH on 4-pentenoate inhibition of growth. Cells were cultured in tryptone broth buffered with Sorensen's phosphate with or without addition of 4-pentenoate (10 mM). Growth was measured as turbidity, and per cent inhibition was calculated after 12 hr of incubation at 37 C. Symbols:* (●) *parent cultured without pentenoate;* (○) N_3 *cultured in presence of pentenoate;* (△) *parent cultured in presence of pentenoate.*

Effect of growth conditions on activity of the uptake system. The effect of growth conditions on the activity of the uptake system is summarized in Table 6. Cells were grown at the expense

TABLE 5. *Effect of pH on uptake of short-chain fatty acids*[a]

Incubation conditions	Pulse (min)	Uptake[b]			
		Parent		N_3	
		*p*H 5	*p*H 7	*p*H 5	*p*H 7
Buffer	1	2.3	.14	2.2	0.72
(^{14}C-butyrate)	5	2.5	.17	2.8	3.8
Glucose	1	2.5	.15	2.8	1.8
(^{14}C-butyrate)	5	2.5	.18	2.8	10.1
Buffer	1	1.8	.21	2.1	0.68
(^{14}C-valerate)	5	1.9	.23	2.0	4.0
Glucose	1	2.1	.27	2.2	1.7
(^{14}C-valerate)	5	2.3	.31	2.5	9.5

[a] The parent and N_3 mutant were grown on acetate, and resting-cell suspensions were prepared as described. Cells were preincubated at 37 C for 10 min in Sorensen's phosphate buffer at *p*H 7 or at *p*H 5, in the presence or absence of glucose (5 mM). ^{14}C-butyrate or -valerate (1 mM) was added, and radioactivity taken up in 1 and 5 min was determined.

[b] Expressed as nanomoles of fatty acid taken up in the time period indicated per milligram of wet cells.

TABLE 6. *Effect of growth substrate on short-chain fatty acid uptake activity*[a]

Growth substrate	Uptake activity[b]	
	Butyrate	Valerate
Acetate	1.48	1.21
Glucose	1.32	1.07
Oleate	1.67	1.13
Butyrate	3.37	2.05
Valerate	3.25	2.37

[a] N_3 was grown at the expense of the carbon source indicated and resting-cell suspensions were prepared. Cells were preincubated at 37 C for 10 min in mineral salts containing glucose (5 mM), ^{14}C-butyrate or -valerate (1 mM) was added, and radioactivity taken up in 1 min was determined.

[b] Expressed as nanomoles of fatty acid taken up in 1 min per milligram of wet cells.

of the carbon source indicated, preincubated in the presence of glucose, and pulsed with ^{14}C-butyrate or -valerate. The transport system was not repressed by growth on glucose nor derepressed by growth on oleate. Butyrate- and valerate-grown cells were comparable and exhibited approximately twice the uptake activity of acetate cells with respect to both the C_4 and C_5 acids. The uptake system is considered constitutive in N_3.

The above data are in contrast to the behavior of the D_1B and D_1V mutants derived from D_1 (*see* Table 1). As shown in Table 7, when D_1B or D_1V was grown at the expense of acetate, the cells exhibited only a slightly higher rate of uptake of short-chain acids than did the D_1 parent. In contrast, when D_1B was grown at the expense of butyrate, cells were induced for both C_4 and C_5 acid uptake. D_1V, when grown at the expense of valerate, was induced for C_5 but not for C_4 acid uptake. Thus, synthesis of the uptake system in these mutants is derepressed by growth on short-chain acids. The data are consistent with the findings (*see* Table 1) that N_3 is pentenoate-sensitive when grown on acetate, whereas D_1B and D_1V are pentenoate-resistant on acetate but pentenoate-sensitive when grown at the expense of butyrate and valerate, respectively.

The growth characteristics of the D_1B, D_1V, and N_3 mutants on butyrate and valerate are compared in Table 8. N_3 grows on the C_4 and C_5 acids with no significant lag; this is consistent with the finding that the uptake system is constitutive in this mutant. In contrast, D_1B and D_1V are inducible for uptake and show a lag of 4 to 6 hr before initiating exponential growth on butyrate and valerate, respectively. D_1B exhibits a long lag before growth begins at the expense of valerate. When D_1B is grown first on butyrate and then is transferred to valerate, this lag is still apparent. This is not understood, because this mutant, when grown on butyrate, exhibits uptake of both the C_4 and C_5 acids. The fact that D_1V does not grow on butyrate is consistent with the uptake data.

TABLE 7. *Uptake of short-chain fatty acids by the D_1 type fatty acid mutants*[a]

Cell type	Growth conditions	Pulse (min)	Butyrate uptake[b]	Valerate uptake[b]
D_1	Acetate	1	0.17	0.21
		5	0.20	0.25
D_1B	Acetate	1	0.23	0.52
		5	0.45	0.64
D_1B	Butyrate	1	3.7	3.2
		5	14.2	11.4
D_1V	Acetate	1	0.27	0.56
		5	0.31	0.96
D_1V	Valerate	1	0.31	1.6
		5	0.35	6.7

[a] Cells were grown at the expense of the carbon source indicated and resting-cell suspensions were prepared. Cells were preincubated at 37 C for 10 min in mineral salts containing glucose (5 mM), ^{14}C-butyrate or -valerate was added, and radioactivity taken up in 1 and 5 min was determined.

[b] Expressed as nanomoles of fatty acid taken up in the time period indicated per milligram of wet cells.

TABLE 8. *Growth characteristics of the D_1B and D_1V mutants*[a]

Cell type	Butyrate		Valerate	
	Lag	α	Lag	α
N_3	0	.23	0	.21
D_1	---	---	---	---
D_1B	4-6	.21	24-30	.13
D_1V	---	---	4-6	.16

[a] Cells precultured on acetate were transferred to mineral salts containing the fatty acid (20 mM) indicated. Lag, time in hours before initiation of exponential growth; α, specific growth rate (*see* reference 13); 0, no significant lag; - - -, no growth in 120 hr.

Uptake of medium-chain fatty acids. Medium-chain fatty acids support growth of N_3 and D_1 but not the parent strain (*see* Table 1). In contrast to short-chain acids, medium-chain acids inhibit growth of the parent strain at *p*H 7. Degree of inhibition is greatest with C_8 and C_9 and least with C_6 and C_{11} (13). As shown in Table 9, the parent strain is permeable to medium chain acids at *p*H 7, but entry is not stimulated by preincubation of cells with glucose. Ability to penetrate the parent strain increases with chain length, hexanoate being similar to valerate. The amount of radioactivity taken up in the presence of azide also increases with chain length and reflects a progressive increase in nonspecific adsorption. As expected, in N_3, uptake of medium-chain fatty acids is energy-dependent.

Table 10 shows the effect of addition of various fatty acids on uptake of nonanoate by N_3 and the parent. In N_3, C_9 uptake is stimulated by glucose but inhibited by acetate. As previously noted, acetate probably inhibits fatty acid uptake by competing for CoA. Uptake of nonanoate is weakly inhibited by butyrate but strongly inhibited by octanoate, indicating that medium- and short-chain fatty acids are taken up by different uptake systems. In the parent strain, nonanoate penetrates but does not appear to be activated as the acyl-CoA ester, and entry is not stimulated by glucose or inhibited by acetate. Penetration is, however, somewhat inhibited by octanoate though not by butyrate.

TABLE 9. *Uptake of medium-chain fatty acids*[a]

Fatty acid	Parent uptake activity[b]			N_3 uptake activity[b]		
	No addition	Glucose	Azide	No addition	Glucose	Azide
Hexanoate	.23	.25	.17	0.35	0.89	.18
Heptanoate	.53	.69	.30	0.90	2.37	.35
Octanoate	.65	.70	.40	0.95	2.05	.41
Nonanoate	.88	.88	.44	1.01	2.91	.44

[a] Cells were grown on acetate, and resting-cell suspensions were prepared. Cells were preincubated at 37 C for 10 min in mineral salts in the presence or absence of 5 mM glucose or azide, the *l*-^{14}C fatty acids indicated (1 mM) were added, and radioactivity taken up in 1 min was determined.

[b] Expressed as nanomoles of fatty acid taken up in 1 min per mg of wet cells.

TABLE 10. *Effect of addition of unlabeled fatty acids on uptake of nonanoate*[a]

Cell type	Unlabeled substrate	Uptake[b]	
		1 min	5 min
N_3	None	0.77	2.7
	Glucose	2.3	9.4
	Acetate	0.47	1.5
	Butyrate	0.67	1.9
	Octanoate	0.26	0.85
Parent	None	0.47	0.87
	Glucose	0.52	0.85
	Acetate	0.47	0.87
	Butyrate	0.54	0.77
	Octanoate	0.38	0.48

[a] Cells were grown on acetate, and resting cell suspensions were prepared. Cells were preincubated at 37 C for 9 min in mineral salts, and the unlabeled substrate indicated (5 mM) was added. After 1 min, ^{14}C-nonanoate (1 mM) was added, and radioactivity taken up in 1 and 5 min was determined.

[b] Expressed as nanomoles of fatty acid taken up in the time period indicated per milligram of wet cells. The amount of radioactivity taken up in the presence of azide was subtracted from the total to give the values reported.

DISCUSSION

In *E. coli* K-12, expression of the β-oxidation operon is repressed during growth at the expense of acetate, but is derepressed by growth on long-chain acids. It is not known whether the regulatory gene product and its interaction with long-chain fatty acid inducers acts in a positive or a negative fashion in causing derepression of the operon. A single regulatory gene product appears to control the synthesis of the β-oxidation enzymes and of a long-chain and a medium-chain fatty acid activation system. The uptake system for short-chain fatty acids is not regulated coordinately with the β-oxidation operon but appears to be controlled by a distinct regulatory gene. Mutants permanently derepressed for the β-oxidation operon concentrate medium-chain but not short-chain acids.

In both the parent and mutant strains, at *p*H 5, short-chain acids penetrate but are not metabolized and inhibit growth. Entry is not energy-dependent, and the substrates appear not to be activated as acyl-CoA esters. A calculation was performed to determine whether the amount of short-chain acid taken up at *p*H 5 could be accounted for by passive equilibration. Taking as average values 2.5×10^{-9} moles taken up per mg of wet cells and 2.5×10^{-9} cells per mg of wet weight, there are 10^{-18} moles of fatty acid taken up per cell. Assuming an individual cell volume of 10^{-12} ml, the internal concentration is 10^{-6} moles per ml or 1 μmole per ml, which is equivalent to the initial external concentration of fatty acid. Thus, at *p*H 5, short-chain acids penetrate presumably as undissociated acids by diffusion.

In contrast, at *p*H 7, the parent strain is not permeable to butyrate and valerate. This is also the case for the D_1, β-oxidation-constitutive mutant. The N_3 mutant is able to concentrate and metabolize short-chain acids at *p*H 7. Mutants derived from N_3 which have a lesion in the β-oxidation pathway and do not grow at the expense of fatty acids C_4 to C_{18} retain the ability to take up short-chain acids (13). Our data indicate that β-oxidation-constitutive mutants do not utilize short-chain acids because they are unable to take up these substrates. A mutation is required for cells to synthesize the uptake system. The gene(s) controlling synthesis of the uptake system are not regulated coordinately with the β-oxidation operon.

An interesting aspect which requires further investigation is the finding that there are two types of mutants with respect to short-chain fatty acid uptake. N_3 synthesizes the uptake system constitutively and is pentenoate-sensitive when grown at the expense of acetate. In contrast, D_1B

and D_1V are inducible for the uptake system and are pentenoate-resistant when grown on acetate but pentenoate-sensitive when grown at the expense of short-chain acids. We suggest as a working hypothesis that synthesis of the uptake system for short-chain acids is controlled by a regulatory gene product. It is proposed that, in the parent strain, this product *does not* interact with short-chain acid inducers, and synthesis of the uptake system is repressed. In the inducible D_1B and D_1V strains, a mutation has occurred such that a product is formed which does interact with the inducers to derepress the uptake system. In the constitutive N_3 strain, a mutation has occurred such that a product is formed which does not repress synthesis of the uptake system. It must be emphasized that this is only a working hypothesis and that we have not investigated the mechanism of regulation of synthesis of the uptake system.

The mutant types also differ with respect to specificity of short-chain fatty acid uptake. N_3 is constitutive for uptake of both butyrate and valerate. The fact that in N_3 butyrate inhibits uptake of ^{14}C-valerate suggests that these substrates are taken up by a system which has at least one component in common. Unsaturated C_4 and C_5 acids also compete with butyrate, suggesting that both unsaturated and saturated short-chain acids are taken up by this system. The 2-methyl- and 2-hydroxy- or amino-substituted C_4 and C_5 acids are not taken up by this system. In contrast to the behavior of N_3, it has been shown that D_1V grows on valerate but not on butyrate. Moreover, when this mutant is grown at the expense of valerate, there is uptake of the C_5 but not the C_4 acid. The behavior of this mutant suggests that the uptake system has a component which exhibits specificity with respect to the C_4 and C_5 acids. D_1B grows on butyrate but exhibits a long lag before growth is initiated at the expense of valerate. This is also the case when cells are grown first on butyrate and then transferred to valerate. When grown on butyrate, however, D_1B exhibits uptake activity for both butyrate and valerate, and thus the long lag exhibited before growth is initiated on valerate is not caused by a deficiency in uptake. The behavior of this mutant has not been explained.

The biochemical nature of the uptake system has not been resolved. As a working hypothesis which is in accord with the data presented, it is proposed that the uptake system is a complex, the synthesis of which is controlled by a regulatory gene and which consists of two components: (i) a diffusion component and (ii) an activation component or components. The diffusion component is required for permeation of the fatty acid anion at *p*H 7 but not for penetration of the undissociated acid at *p*H 5. The activation component effects acylation of the fatty acid as the acyl-CoA ester, and energy is required for this process. During growth on short-chain fatty acids, the β-oxidation process provides energy required for uptake. The activation component(s) appear to exhibit specificity for the C_4 and C_5 acids, respectively. Recently, Klein et al. (6) described an uptake system for medium- and long-chain acids in *E. coli* K-12 strain Y mel. β-Oxidation-constitutive mutants concentrated fatty acids C_8 and higher. Hexanoate was taken up at a low rate and butyrate not at all. The ability to take up and to grow at the expense of fatty acids paralleled the in vitro specificity of the fatty acid acyl-CoA synthetase formed by this strain. The inability of mutants which lack the synthetase to take up these substrates indicates that activation is an integral part of uptake. The authors suggest the term *vectoral acylation* of fatty acids by analogy with vectoral phosphorylation of sugars.

The medium-chain uptake system in the K-12 strain employed here is similar to that described by Overath's group (6) except that hexanoate is taken up. The fact that N_3 shows less uptake of the C_6 than of the C_7 to C_9 acids correlates with the lower specific growth rate shown by β-oxidation-constitutive mutants on hexanoate. The medium-chain uptake system does not concentrate the C_4 and C_5 acids. In the parent strain cultured on acetate at *p*H 7, medium-chain acids (C_7 to C_{10}) penetrate and inhibit growth. It is likely that under these conditions the fatty acids are not activated as the acyl-CoA ester. In N_3, but not in the parent strain, incubation in the presence of glucose stimulates uptake of medium-chain acids. It has not been determined experimentally in this strain whether medium-chain and long-chain acids are concentrated by a common or by different uptake systems, but growth data (13) suggest the latter.

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ADDENDUM IN PROOF

Recent studies by E. Vanderwinkel, M. de Vlieghere, and J. Vande Meerssche (Eur. J. Biochem. **22**:115–120) confirm and extend these observations.

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