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Function of the Glyoxylate-condensing Enzymes

I. Growth of *Escherichia coli* on *n*-Valeric Acid

PHILIP FURMANSKI, WARNER S. WEGENER, HENRY C. REEVES,[1] AND SAMUEL J. AJL

Department of Biochemistry, Research Laboratories, Albert Einstein Medical Center, Philadelphia, Pennsylvania 19141

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Growth of *Escherichia coli* E-26 on valeric acid results in the formation of a mutant population characterized by the ability to form constitutively several glyoxylate-condensing enzymes. This mutant also differs from the parent organism in the ability to effect rapid growth on a series of short-chain fatty acids. These mutants were utilized in postulating genetic relationships among the various glyoxylate-condensing activities and also in correlating the presence of these enzymes with the ability of the mutants to initiate growth quickly on short-chain fatty acids.

Malate synthase (E.C.4.1.3.2) and isocitrate lyase (E.C.4.1.3.1) have been shown to be required for the growth of *Escherichia coli* with acetate as the sole carbon source (1). Although a series of metabolic routes, initiated by the condensation of other short-chain fatty acid acyl-coenzyme A (CoA) esters with glyoxylate have been described (3) and the enzymes differentiated (8), their role in growth is still being elucidated. Whereas the specific activities of these enzymes are high when cells are grown with valerate or heptanoate as the carbon source, low activity is observed after growth on propionate, caproate, acetate, or glucose (W. S. Wegener, P. Furmanski, and S. J. Ajl, Bacteriol. Proc., p. 104, 1966).

It has been reported that the growth of *E. coli* on valerate results in the formation of a mutant population which is constitutive for the glyoxylate-condensing enzymes (6). These mutants appear consistently under these conditions and are preceded by an intermediate population of valerate-"adapted" cells, which are not constitutive for these enzymes. The mutant cells are capable of growth on a number of short-chain fatty acid substrates after a very short lag period, whereas both the parent and adapted cells exhibit either a long lag period or will not grow at all. The data presented here provide an insight into the mechanism by which growth on valerate results in the formation of the mutant population, and suggest that there is a correlation between the activity of the glyoxylate-condensing enzymes and the ability of an organism to utilize various short-chain fatty acids as the sole carbon source.

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MATERIALS AND METHODS

E. coli, strain E-26, was maintained on Trypticase Soy Agar and grown as specifically described in mineral-salts medium (4) containing 0.2% (w/v) of the particular carbon source. All acids employed as carbon source were used as the sodium salts and were of commercial analytical grade purity.

Valeric acid (Eastman Chemicals, Rochester, N.Y.) was purified by fractional distillation and gas chromatography, as described (6). This purified material yielded the same results as the commercial reagent and therefore the latter was used in all growth studies.

Cells were grown at 37 C on a Gyrotory (New Brunswick Scientific Co., New Brunswick, N.J.) shaker with 100 ml of medium in 300-ml baffled shake flasks or with 8 liters of medium in 10-liter carboys with forced aeration. Petri plates were prepared with the same mineral-salts medium containing 1.5% Noble agar. Growth studies were conducted in 500-ml Klett flasks containing 50 ml of medium on a Gyrotory shaker at 37 C, and turbidity was measured at 660 mμ by using a Klett-Summerson photoelectric colorimeter. Turbidity is expressed as optical density (OD).

For enzyme assays, cells were harvested at 4 C by centrifugation at 5,000 × *g*. Cell-free extracts were prepared and enzyme activities were determined as previously described (7). Enzyme activity is expressed as millimicromoles of glyoxylate utilized per 15 min per milligram of protein.

Radioactivity was determined by placing samples in 10 ml of scintillation fluid consisting of 0.4% 2,5-bis-[2-(5-*t*-butylbenzoxazoyl)]-thiophene (Packard Instrument Co., Inc., Downers Grove, Ill.) in toluene-absolute ethyl alcohol (2:1, v/v) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Growth characteristics of cells grown in valerate medium. E. coli, strain E-26Val, was obtained by inoculating the parent strain E-26 into 100 ml of mineral salts-valerate medium and incubating with shaking at 37 C for 5 days. Samples of this culture were then streaked on mineral salts-valerate-agar plates and incubated at 37 C for 24 to 48 hr. Isolated colonies (hereafter designated as *E. coli*, strain E-26Val) were selected, streaked on valerate-mineral salts slants, incubated 24 to 48 hr at 37 C, and stored at 4 C. Strain E-26But was obtained in a similar fashion after growth in mineral salts-butyrate medium.

To compare the growth characteristics of strain E-26Val and E-26But with the parent culture, the appropriate strain was inoculated into tubes containing 10 ml of Trypticase Soy Broth and incubated at 37 C for 6 to 8 hr. The tubes were then centrifuged, the cells were washed with sterile mineral-salts solution, and a sample was inoculated into 50 ml of mineral-salts medium containing 0.2% of a single carbon source in 500-ml Klett flasks. Strain E-26, which had been maintained on Trypticase Soy Agar slants, was treated in an identical manner. The results in Table 1 show that valerate-grown cells (strain E-26Val) have a definite growth advantage on short-chain fatty acids. This characteristic of strain E-26Val is stable and is not lost even after repeated subculture on complex media (6). It is further noted in Table 1 that cells isolated after growth on butyrate (strain E-26But) also exhibit a reduced lag period when grown in butyrate, valerate, heptanoate, or caproate medium. In contrast to E-26Val, however, the E-26But strain does not exhibit a reduced lag in propionate medium. The fact that the capacity of this strain to grow, after a short lag period, is not repressed by extensive subculture in complex media, is evidence that E-26But represents a mutant population. Thus, growth of *E. coli* E-26 on valerate, as well as on butyrate, results in a genotypically altered population.

TABLE 1. *Comparison of ability of Escherichia coli mutants to grow on short-chain fatty acids*

Cell type	Lag period (hr)[a] before growth				
	Propionate	Butyrate	Valerate	Heptanoate	Caproate
E-26.....	34	250[b]	130	77	180
E-26Val...	25	5	5	17	22
E-26But...	39	29	32	46	86

[a] Time required for culture to reach a Klett reading of 10 from an initial reading of 0 to 1.

[b] Varied from 12.5 to 16 days.

Role of the adapted cell. In a previous report (6), it was shown that in a valerate culture two types of cells could be isolated, a valerate mutant and a valerate-adapted cell. The latter, after subculture on complex media, regained several characteristics of the parent, i.e., long lag periods before growth on fatty acids and very low activity of the glyoxylate-condensing enzymes. Adapted cells differ from mutants (E-26Val) in that they give rise to small colonies when plated on valerate agar, whereas the mutants yield large colonies (6). No difference in colony size between the two types of cells is evident on Trypticase Soy Agar or on acetate-agar, on which both types produce large colonies.

Pure cultures of adapted cells (i.e., by the criteria of glyoxylate-condensation enzyme levels, growth characteristics, and colony size) were prepared by inoculating valerate medium with a high inoculum of E-26 cells (initial OD of 0.04 to 0.06), harvesting the culture in the early logarithmic phase of growth, and selecting isolated colonies. When these adapted cells were incubated in acetate medium (1.2 ml each in 40 screw-capped test tubes) for 48 hr, the colony count on Trypticase Soy Agar increased from 10^6 to 2×10^9/ml. Plating samples of these independent cultures on valerate gave a distribution of large colonies of up to 100%, with very few plates yielding no large colonies. Multiple samples from the same culture gave good agreement. Thus, after E-26 cells have become adapted to growth on valerate, they subsequently give rise to mutants. Further, a determination of the rate of spontaneous mutation of E-26 → E-26Val by a Luria-Delbrück fluctuation analysis (2) gave an upper limit value of 5×10^{-10}.

Since the appearance of mutants from adapted cells appears to differ from the direct appearance of mutants from the parent, the effect of valeric acid may be related to a change in the population which occurs either before or concomitant with the appearance of adapted cells, i.e., during the lag period. To investigate the nature of this change, the following experiment was performed.

Strain E-26 cells were inoculated into Klett flasks containing 50 ml of valerate medium and were incubated with shaking. Samples were removed at various times, inoculated onto a Trypticase Soy Agar slant, incubated for 12 to 24 hr, then transferred and grown in Trypticase Soy Broth to repress the ability of adapted cells to grow on valerate. After 6 hr of incubation, cells were harvested by centrifugation, washed, and inoculated into tubes of valerate or butyrate

medium (10 ml in a screw-capped test tube). The results are presented in Fig. 1.

Under these conditions, cell samples removed from the original valerate culture during the exponential phase of growth grew quickly when subcultured in either valerate or butyrate medium. This is in accord with the observation that samples removed during exponential growth exhibit a progressive increase in the proportion of mutant cells in the population (8). This has been demonstrated by plating sequential samples during exponential growth on valerate-agar and determining the percentage of large (mutant) colonies. In contrast, the initial growth curve samples (those incubated in valerate medium from 0 to 160 hr) exhibit a progressive increase in ability to grow on valerate, but exhibit no growth advantage on butyrate.

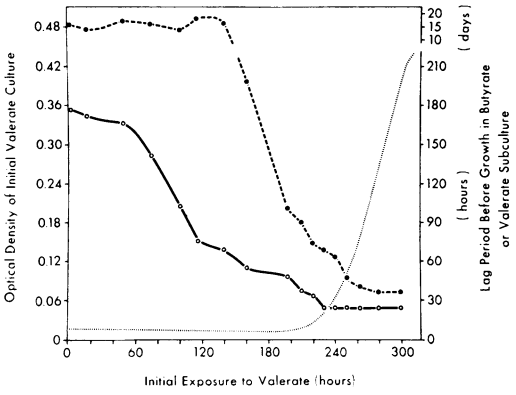

FIG. 1. *Cumulative valerate effect on Escherichia coli E-26. Dashed line and solid line represent the time required for the given samples to grow on subsequent subculture in butyrate or valerate media, respectively; (·····) the growth curve from which the appropriate samples were removed.*

When the distribution of adapted cells and mutants in these cultures was examined after a single transfer to Trypticase Soy Broth, it was found that there were only small colonies. When samples were taken after growth in the valerate tubes, however, the distribution of cell types shifted considerably, so that there were predominantly large colonies (mutants). This pattern followed the growth curve. There is no evidence of mutants in the early samples placed in butyrate, but growth on valerate was enhanced and the proportion of mutants increased. Thus, mutagenesis and growth may be a function of the formation of adapted cells. Further, cells exposed to valerate have a progressively higher frequency of conversion to the mutant cell, specifically in the presence of valerate.

Since adapted cells have the capacity to grow on valerate, however, it was necessary to ascertain whether growth per se in the presence of this substrate was sufficient to raise the mutation frequency or rate. To investigate this possibility, strain E-26, previously grown in Trypticase Soy Broth, was inoculated into either valerate or butyrate medium (50 ml in a 500-ml Klett flask) and limiting acetate was added (to a concentration of 0.02%) to the cultures at various times. The results shown in Fig. 2 indicate that growth per se in the presence of valerate cannot explain the observed mutagenesis, because the pattern of growth remained unchanged. In the butyrate cultures with greater numbers of cells, growth occurred earlier, but there was not necessarily a relationship between the incubation time and the appearance of mutants (growth). This is in accord with the hypothesis that butyrate mutagenesis is a classical spontaneous event. Similar results were obtained when the valerate or butyrate medium

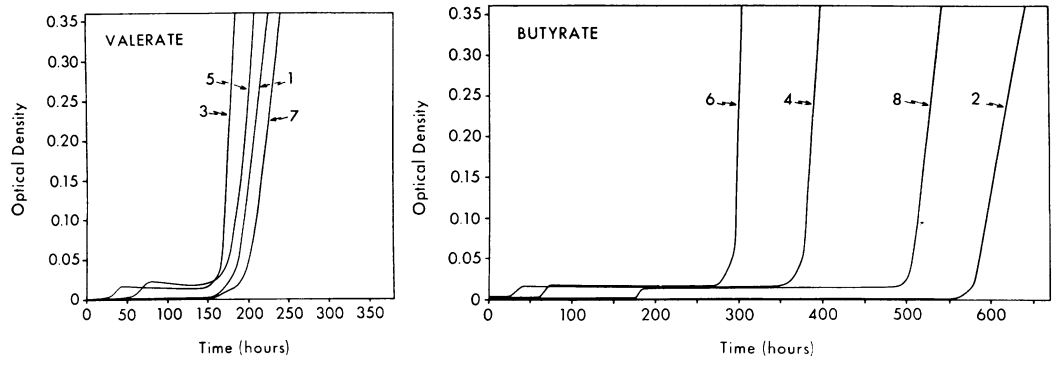

FIG. 2. *Effects of growth in the presence of valerate and butyrate on mutagenesis. Acetate added to a final concentration of 0.02%: culture 1, none added; culture 3, 0 hr; culture 5, 48 hr; culture 2, none added; culture 4, 0 hr; culture 6, 24 hr; culture 8, 165 hr.*

was supplemented with 0.04% acetate or with 0.02% yeast extract.

To ascertain whether the mutagenesis on valeric acid medium was specific, the following experiments were performed. Strains E-26 and E-26Val were grown overnight in Trypticase Soy Broth. The cells were harvested and tested for their susceptibility to penicillin, streptomycin, erythromycin, and tetracycline. Identical results were obtained with the two strains. It has also been previously reported that the parent and mutant were similar in the activities of a number of enzymes (6).

Permeability studies. If adapted cells and mutants differed from the parent in their permeability to valerate and if adapted cells were formed normally in response to valerate, this might explain the specificity of valerate and the subsequent high frequency of occurrence of E-26Val mutants. Only when the cells became permeable (adapted) could the selective effect of valerate become apparent.

The permeability of strains E-26 and E-26Val to valerate-*1*-^{14}C and acetate-*1*-^{14}C was compared. The organisms were grown in acetate broth for 18 hr, centrifuged, washed, and adjusted to an OD value of 1 (660 mμ) with mineral-salts solution. A 0.2-ml amount of this suspension was added to 1.0 ml of mineral-salts medium containing 2 mg of ^{14}C-labeled carbon source (specific activity of 0.1 μc/mg) in 25-ml Erlenmeyer flasks. The flasks were shaken in a Dubnoff metabolic shaker at 37 C. After various time intervals, 10 ml of cold mineral-salts solution was added and the suspension was immediately filtered through a 25-mm filter (0.45 μ pore size; Millipore Corp., Bedford, Mass.). The filters were rapidly washed with 5 ml of cold mineral-salts solution and radioactive uptake was determined. The results presented in Table 2 show that uptake of *1*-^{14}C-labeled acetate is the same in strains E-26 and E-26Val. In contrast, the uptake of valerate-*1*-^{14}C by strain E-26Val is considerably higher than with E-26. However, the uptake in E-26 is still five times that of heated cells.

The difference in valerate uptake between the two strains is considered to be due to a difference in metabolic ability, because E-26Val can utilize valerate without a lag, but E-26 cannot. One possibility is that the lower uptake in E-26 is due to a heat-labile adsorption. Another possibility is that strain E-26Val is able to incorporate the valerate into cellular constituents, whereas E-26 cells cannot metabolize the compound. Under the latter conditions, the uptake may reflect an equilibration with the external valerate. Thus, there would be no real difference in permeability between the two strains. This possibility is favored.

Glyoxylate-condensing enzyme activity in strains E-26 and E-26Val. Strains E-26 and E-26Val were grown with a number of different carbon sources in 2-liter baffled flasks containing 1-liter quantities of medium. Cell-free extracts were prepared and the activities of the glyoxylate-condensing enzymes were determined as described (7).

The results presented in Table 3 show that E-26 cells do not possess appreciable activities of α-hydroxyglutarate synthase or β-ethylmalate synthase and only a low level of β-*n*-propylmalate synthase when grown under the conditions indicated. In contrast, strain E-26Val cells contain high activities of these enzymes under the same conditions of growth. It should be particularly noted that E-26Val cells possess high enzyme activities even when grown in highly repressive media such as that containing glucose. These data suggest that E-26Val is a permanently derepressed mutant of E-26 with respect to the glyoxylate-condensing enzymes, and, further, that strain E-26 is constitutive for malate synthase.

The enzymatic activities in E-26Val do not appear to be due to a structural mutation in malate synthase, because these enzymes may be differentiated by heat inactivation and patterns of appearance during growth on various sub-

TABLE 2. *Permeability of Escherichia coli mutants*

Substrate	E-26[a]			E-26Val[a]		
	30 sec	60 sec	120 sec	30 sec	60 sec	120 sec
Acetate-*1*-^{14}C	673 (55)	876 (71)	1,097 (82)	785 (64)	919 (75)	1,185 (96)
Acetate-*1*-^{14}C[b]	118 (10)	106 (9)	112 (9)	71 (6)	97 (8)	113 (9)
Valerate-*1*-^{14}C	587 (32)	556 (30)	648 (35)	1,006 (54)	1,541 (83)	2,734 (147)
Valerate-*1*-^{14}C[b]	129 (7)	117 (6)	130 (7)	95 (5)	80 (4)	135 (7)

[a] Results are shown in counts per minute. Uptake in millimicromoles is given in parentheses.
[b] Cells heated at 100 C for 10 min.

TABLE 3. *Glyoxylate-condensing activities of Escherichia coli E-26 mutants*

Cell type	Growth substrate	Specific activity[a]			
		Malate synthase	α-Hydroxyglutarate synthase	β-Ethylmalate synthase	β-*n*-Propylmalate synthase
E-26	Acetate	63.9	—[b]	—	12.9
	Propionate	60.7	4.1	3.0	10.7
	Glucose	48.7	—	—	3.2
E-26Val	Acetate	64.5	39.1	41.6	58.7
	Propionate	61.1	45.6	39.6	56.6
	Glucose	50.1	29.6	22.3	45.1

[a] Millimicromoles of glyoxylate utilized per 15 min per mg of protein.
[b] Represents no detectable activity.

strates (8). Further, low activities of these enzymes are present even in the absence of constitutive mutation, for example, following growth on propionate.

Effect of the glyoxylate-condensing enzymes on properties of valerate-grown cells. In these investigations we isolated E-26Val strains which had lower than normal levels of α-hydroxyglutarate synthase constitutively, and which were unaffected in their ability to catalyze the other glyoxylate-condensing reactions. The ability of one of these mutants to grow on propionate is compared to the normal in Fig. 3. It can be seen that the strain containing lower enzyme activity required a longer lag period on this substrate than did the strain with the higher activity. But both strains grew at comparable rates when other short-chain fatty acids were employed as substrates.

It is suggested that the decrease in the activity of this enzyme is due to a structural mutation in α-hydroxyglutarate synthase. This would provide further evidence for the differentiation of the glyoxylate-condensing enzymes, since the other activities are unaffected by this mutation. It also provides a correlation between the two E-26Val properties, high activity of condensing enzymes and short lag before growth on fatty acids.

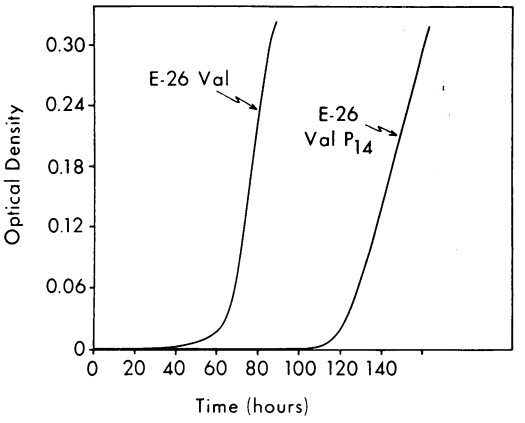

FIG. 3. *Relationship between activity of α-hydroxyglutarate synthase and growth on propionate. The specific activity of α-hydroxyglutarate synthase under these conditions was 35.8 for E-26Val and 15.0 for E-26Val P14.*

DISCUSSION

Growth of *E. coli* E-26 on valerate as the sole carbon and energy source is preceded by a long lag period. During this lag, valerate exerts some specific effect on the cells, presumably inducing the enzymes necessary for growth. This results in the phenotypic alteration of the cell population in such a way that the cells are capable of growth on valerate. However, these cells have also changed genotypically and are capable of acquiring certain characteristics constitutively (mutagenesis) with a rather high frequency. The alterations are a function of valeric acid specifically, and, after incubation in the presence of valeric acid, are maintained as a stable characteristic. Subsequent growth on complex media where selectivity is no longer a factor and where repression of a normal adaptive response would be expected, has no effect.

The explanation of the phenomenon of growth on valerate is probably a function of a combination of events and may involve a number of mutations and discrete steps. One of these events, which occurs early in the process, is dependent on valerate to produce the adapted cell. The sequence leading to the mutant from the adapted cell does not appear to be a totally selective process, since the nature of growth on valerate does not correspond to a pattern due to a spontaneously arisen mutant. On the other hand,

growth on butyrate seems to occur via a spontaneous mutation at low frequency.

From the data in Table 2, and assuming that there is a point mutation with respect to the glyoxylate-condensing enzymes involved in the mutagenesis on valeric acid, it is possible to make certain predictions. The lesion has affected the levels of α-hydroxyglutarate synthase and β-ethylmalate synthase much more than β-*n*-propylmalate synthase. In addition, the ratios of the increased enzyme levels, expressed as a result of the mutation, are relatively constant. There is a certain constitutive level of β-*n*-propylmalate synthase associated with the parent cell, which is proportional to the level of malate synthase. Since the parent is known to be constitutive for malate synthase, these observations suggest that there are two β-*n*-propylmalate synthases, one genetically associated with malate synthase and one on an operon also describing the activities of α-hydroxyglutarate synthase and β-ethylmalate synthase.

It is known that there are two malate synthases in *E. coli*. One enzyme is associated with the functioning of the glyoxylate bypass in an anaplerotic sequence and the other is concerned with its catabolic role in the dicarboxylic acid cycle of glycolate catabolism (5). Thus, one of the malate synthases may be located on the proposed operon for α-hydroxyglutarate synthase, β-ethylmalate synthase, and one of the β-*n*-propylmalate synthases, whereas the other malate synthase is coordinately regulated with the second β-*n*-propylmalate synthase, implying functional relationships within the enzyme groups yet to be elucidated. Evidence in support of this hypothesis is the observation that during growth on glycolate there is a shift from the anaplerotic malate synthase to the catabolic malate synthase and simultaneous induction of the other glyoxylate-condensing enzymes (*unpublished data*).

Valerate mutants are permanently derepressed for the enzymes catalyzing the condensation of glyoxylate with various short-chain fatty acid acyl-CoA esters. These mutants possess a definite selective advantage with respect to growth on short-chain fatty acids as the sole carbon source. It is, therefore, tempting to postulate that the significant decrease in the lag period preceding the growth of the mutant (E-26Val), compared with the parent (E-26), may be due to the presence of these glyoxylate-condensing enzymes. The activity of these enzymes might permit the early accumulation of intermediates or the production of energy, or it might serve as part of a major catabolic or anabolic route during growth.

This correlation is, of course, not yet definitive, because the true nature of the mutants and the nature of the normal metabolic routes still available in the mutant must be elucidated. However, comparison of two mutants, which were derived identically and differ only in the constitutive levels of the enzymes condensing glyoxylate with propionyl-CoA to yield α-hydroxyglutarate, shows that there is a correlation between the length of the lag period and the activity of the enzymes. Thus, the initiation of growth on propionate may be dependent on the intracellular level or availability of C_4 acids, such as succinate, to the cell, and this mechanism may be reflected in the decreased lag period observed in the mutant.

The quantitative correlation of in vitro levels of enzymes with an in vivo effect is an intriguing study, and there is evidence that a similar phenomenon will be apparent with the glyoxylate-butyryl-CoA condensation (β-ethylmalate synthase) and growth on butyrate. The fact that these differences appear only in the growth substrate corresponding to the enzymatic substrate lends credence to the hypothesis. Since these mutants are constitutive for all the enzymes in question, this may not be the true functional significance of the enzymes, but it does support the idea that they have a significant role in the growth of *E. coli* on fatty acids. Experiments to elucidate the physiological function of these enzymes are currently being undertaken.

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