JOURNAL OF BACTERIOLOGY, Nov. 1969, p. 601-604
Vol. 100, No. 2

Evidence for Two Genes Specifically Involved in Unsaturated Fatty Acid Biosynthesis in *Escherichia coli*

JOHN E. CRONAN, JR., CLAIRE H. BIRGE, AND P. ROY VAGELOS
Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri 63110

Received for publication 5 September 1969

Unsaturated fatty acid auxotrophs of *Eschericha coli* have been divided into two distinct cistrons by extract complementation and genetic complementation based on abortive transduction. Lesions in one cistron result in the loss of the β-hydroxydecanoyl thioester dehydrase which produces the first biosynthetic intermediate in unsaturated fatty acid formation. Evidence is presented which indicates that lesions in the second cistron result in the lack of a second enzyme specifically involved in the biosynthesis of unsaturated fatty acids.

Auxotrophs of *Escherichia coli* requiring an unsaturated fatty acid were first isolated in this laboratory (17) and have since been reported by others (8, 12). The first mutant was characterized (17) as deficient in the β-hydroxydecanoyl thioester dehydrase which is responsible for the formation of *cis*-Δ^3-decenoyl acyl carrier protein (ACP), the first specific intermediate in the biosynthesis of the unsaturated fatty acids of *E. coli* (3).

In this investigation, we studied a collection of unsaturated fatty acid auxotrophs to examine the question of the existence of other enzymes specific for the biosynthesis of unsaturated fatty acids. We demonstrate the existence of two genes involved in unsaturated fatty acid biosynthesis and present evidence that these genes code for enzymes responsible for two different steps in unsaturated fatty acid biosynthesis, one of which is the β-hydroxydecanoyl thioester dehydrase.

MATERIALS AND METHODS

As in the paper of Henning et al. (8), the unsaturated fatty acid auxotrophs will be referred to as oleate auxotrophs although it is realized that this term is inaccurate because oleate is not a normal component of *E. coli* (4) and many other fatty acids will support the growth of these mutants (16).

Bacterial strains. All the strains used are *E. coli* K-12 strains. L010 and L020 are oleate auxotrophs derived from Hfr 139. L010 has been previously described (16, 17). L030 and L040 are oleate auxotrophs derived from strain 8 of Hayashi et al. (7). All these mutants were isolated as previously described (17) and show a growth requirement for an appropriate unsaturated fatty acid. The spectrum of unsaturated fatty acids which will support growth of L010 has been described (16). The other mutants behave in a similar manner although such detailed data are not yet available. UC204 and UC1098 are temperature-sensitive mutants derived from strain W3110. These strains are from the collection of D. L. Wulff of the University of California, Irvine. These mutants are unable to grow at 40 C unless the medium is supplemented with an appropriate unsaturated fatty acid, although growth procedes at 30 C without supplementation. The growth of these mutants is not supported by saturated or hydroxy fatty acids (Wulff and Cronan, *unpublished data*).

Strain 30$^-$, an oleate mutant derived from Hfr 3000 was the gift of C. Fred Fox of the University of Chicago.

Media. The minimal medium used was medium E (18). Oleic acid (100 μg/ml) was used as the unsaturated fatty acid supplement as previously described except that Brij 35 (polyethyleneglycolmonolauryl-ether) detergent was used to solubilize the fatty acid instead of Tween 40. Brij 35 was used at a ratio of 4 μg to 1 μg of oleic acid. Carbon sources were added at 0.2 to 0.4% (glucose, glycerol, and succinate) or 0.1% (oleate). Solid minimal medium was made by addition of 12 g per liter of Noble agar. L-broth and L-broth soft and bottom agar have been described (10). The 56-LP buffer is 0.1 M tris(hydroxymethyl)-aminomethane (Tris)-chloride, *p*H 7.2, 0.01 M KCl, 3×10^{-4} M KH_2PO_4, and the salts of medium 56 (11).

Extract complementation assay. Crude cell extracts were made and assayed for fatty acid synthesis by measuring the incorporation of ^{14}C-labeled malonyl-CoA into fatty acids as previously described (17). The radioactive fatty acids resulting from the incubations were separated into saturated, unsaturated, and hydroxy fatty acid fractions on silver nitrate-impregnated silica gel thin-layer chromatograms (4, 17). The fractions were quantitated by scraping

the appropriate areas of silica gel into vials and counting in a toluene-based scintillation solution. The saturated and unsaturated fractions consisted mainly of stearic and *cis*-vaccenic acids, respectively. Protein concentrations were determined by the biuret reaction (9).

Preparation of phage stocks. Phage P_1kc, hereafter referred to as P_1, was used to mediate transduction. Stocks were obtained by growing the oleate auxotrophs to 10^8 to 2×10^8 cells/ml in L-broth supplemented with oleate, then infecting with a multiplicity of 0.01 after adding $CaCl_2$ to 2.5 mM. The stocks were then aerated until lysis was complete and sterilized with $CHCl_3$. Ethylenediaminetetraacetic acid (2.5 mM) was added to inhibit the adsorption of phage to bacterial debris, and the stock was centrifuged to remove the debris. We were unable to obtain satisfactory phage stocks from L010, L020, and 30^- by growth in liquid medium. Stocks from these mutants were obtained by growth on solid media by the following procedure (D. L. Wulff, *personal communication*). The oleate auxotrophs were grown as above and resuspended in 56-LP at 10^9 cells/ml. One ml of this suspension was added to a mixture of 2.5 ml of molten L-broth soft agar (supplemented with oleate), $CaCl_2$ (2.5 mM), and $1-5 \times 10^6$ P_1 plaque formers in 0.1 ml of L-broth. This mixture was poured onto a warm freshly poured L-broth bottom agar plate. The plates were incubated for 5–6 hours at 37 C and harvested as above. All stocks were titered on *Shigella dysenteriae* strain 16 and ranged from 10^9 to 5×10^{10} plaque formers per ml. The phage stocks were grown on a given mutant at least twice. Most experiments were repeated with two independently derived phage stocks.

Abortive transduction. The various oleate auxotrophs were grown to stationary phase on oleate as carbon source, then diluted 10-fold into L-broth-oleate, and grown to 4×10^8 to 5×10^8 cells/ml. $CaCl_2$ (5 mM) was added, the culture was incubated for 5 min at 37 C, and 0.05 ml of the culture was added to 0.05 ml of 2×10^9 plaque formers/ml of P_1. This mixture was incubated at 37 C for 20 min and then diluted to 1 ml with medium E, and 0.1 ml samples were plated on glucose-supplemented minimal medium. Plates from crosses involving UC204 and UC1098 were incubated at 40 C; the plates from the other crosses were incubated at 37 C. After 2 to 3 days of growth, the plates were examined under a dissecting microscope for the presence or absence of minute colonies. Controls routinely run consisted of the recipient alone, the recipient infected with phage grown on the recipient, and the recipient infected with phage grown on a parental strain, as well as a sterility test of the phage stock.

Materials. Culture media were from Difco. Brij 35 detergent was from Emulsion Engineering, Inc., Elk Grove Village, Ill. Oleic acid was from the Hormel Institute, Austin, Minn.

RESULTS

In vitro complementation. The various oleate mutants contain fatty acid synthetase activity comparable to that of L010 (17). When the products of these reactions were analyzed by thin-layer chromatography, it was noted that all of these mutants were deficient in unsaturated fatty acid biosynthesis (Table 1). When the extracts of these mutants were mixed with an equal amount of an extract from a parental strain, a normal pattern of products was obtained, indicating the lack of an inhibitor of unsaturated fatty acid synthesis in the mutant extract (Table 1). When equal amounts of extracts of various pairs of these mutants were mixed and analyzed, two patterns were found—those pairs which acquired a normal ratio of products and those which retained their mutant character (Table 1).

TABLE 1. *Extract complementation of oleate auxotrophs*[a]

Additions	Extracts						
	L010	L020	L030	L040	30^-	UC204[d]	UC1098[d]
None	0.05[b]	0.24[b]	0.26[b]	0.47[b]	0.33	0.63[b]	0.45[b]
Parental extract[c]	3.43[b]	2.94	3.75	4.10[b]	2.33	2.09	2.00
L010 extract		2.97[b]	2.42	2.63	2.41	1.99	0.37[b]
L020 extract			0.59[b]	0.41	0.45	0.73	1.89[b]
L030 extract				0.50	0.47		1.83

[a] Equal amounts (0.2 mg) of protein from each of two extracts were mixed and incubated in the reaction mixture. After 30 min at 37 C (or 41 C, *see above*), the radioactive fatty acids were extracted, methylated with diazomethane, and analyzed by thin-layer chromatography. Values are the ratios of the total recovered counts per minute in unsaturated fatty acids divided by the total recovered counts per minute in saturated fatty acids.

[b] Average value of two experiments.

[c] The parental strains gave ratios of 2.7 to 3.1 (for Hfr 139, Strain 8, and Hfr 3000) and 2.0 to 2.1 (for W3110 assayed at 41 C) when assayed alone.

[d] Assayed at 41 C. The ratios of UC204 and UC1098 assayed alone at 25 C were 2.4 and 1.6, respectively. The ratios of UC204 and UC1098 at 30 C were 2.1 and 0.87, respectively.

This experiment defined two groups of oleate mutants. One group which we will refer to as fabA (for fatty acid biosynthesis) is composed of mutants L010 and UC1098. The remaining mutants comprise the second group, fabB. The members of a group retained their mutant character when their extracts were mixed and thus were unable to perform in vitro complementation. Strong complementation resulted when the extract of a member of one group was mixed with the extract of a member of the other group. We have no evidence of the existence of additional complementation groups although we have not examined enough mutants to exclude this possibility.

Genetic complementation. Further evidence that the two groups of mutants constitute lesions in different genes was obtained by genetic complementation based on the technique of abortive transduction. Abortive transduction has been extensively used as a complementation test in *Salmonella typhimurium* with phage P22 (5, 13) and less extensively but successfully in *E. coli*-phage P_1 system (1, 2, 6, 15). Complementation of two mutants is denoted by the presence of irregularly shaped minute colonies on minimal plates in addition to the large round macrocolonies formed by recombination (13). Lack of complementation is denoted by the complete absence of such minute colonies. These minute colonies seen in our crosses appear to be classical abortive transductants (13). Of 10 such minute colonies spread on minimal plates, 6 formed only a single minute colony, whereas the remainder formed no minute colonies at all. No macrocolonies were formed on these minimal plates, but when minute colonies were spread on oleate-supplemented media about 20 macrocolonies (and no minute colonies) were formed per minute colony spread. Since a minute colony contains about 10^4 cells, only about 0.1% of the cells in a minute colony were viable. This was expected because oleate auxotrophs die when starved of the fatty acid (8). This complementation test is applicable to the mutants tested except L030 because they gave minute colonies when crossed with phage grown on a parental strain and no minute colonies when crossed with phage grown on the mutant itself (Table 2). L030 was not used as a recipient because mutant cells formed very small colonies on glucose-minimal medium upon prolonged incubation. These colonies were difficult to distinguish from the minute colonies formed by abortive transduction. As can be seen in Table 2, genetic complementation clearly separated the oleate auxotrophs into two groups, the same groups (fabA and fabB) defined by the extract complementation experiments.

DISCUSSION

The evidence presented above demonstrates that there are at least two genes (cistrons) responsible for unsaturated fatty acid biosynthesis in *E. coli*.

Since the fabA and fabB complementation groups both include a mutant with a temperature-sensitive lesion (UC1098 and UC204, respectively), this indicates that both cistrons produce protein products. It, therefore, is very unlikely that either of these cistrons is an operator-type regulatory gene. Both groups of oleate auxotrophs

TABLE 2. *Genetic complementation of oleate auxotrophs by abortive transduction*[a]

Donor	Recipient[b]					
	L010 (A)[c]	L020 (B)	L040 (B)	30⁻ (B)	UC204 (B)	UC1098 (A)
W3110	+	+	+	+	+	+
L010 (A)[c]	−	+	+	+	+	−
L020 (B)	+	−	−	−	−	+
L030 (B)	+	−	−	−	−	+
L040 (B)	+	−	−	−	−	+
30⁻ (B)	+	−	−	−	−	+
UC204 (B)	+	−	−	−	−	+
UC1098 (A)	−	+	+	+	+	−

[a] The donors are stocks of P_1 phage grown on the strain given in the extreme left-hand column. The recipients are strains given in the column heads at the top of the table. A plus sign denotes the presence of 50 to 500 minute colonies among the progeny of a transductional cross. A minus sign denotes the complete absence of minute colonies in the cross.

[b] Mutant L030 could not be used as a recipient due to its "leakiness."

[c] The letter A or B in parentheses after the designation of each mutant denotes the complementation group (fabA or fabB, respectively) of that mutant as derived from extract complementation (Table 1).

are recessive to the parent strain as tested by extract complementation and genetic complementation. This indicates that the phenotype of these mutants is not due to the accumulation of an inhibitor of unsaturated fatty acid biosynthesis or to a lesion in a trans-dominant regulatory gene. One of the fabA mutants, L010, is known to be deficient in the β-hydroxydecanoyl thioester dehydrase, the first enzyme specifically involved in unsaturated fatty acid biosynthesis (17). Thus, we expect that the phenotype of the fabB mutants is due to a mutation in the structural gene for another enzyme specific for unsaturated fatty acid biosynthesis. It seems reasonable that this postulated enzyme could be a species of enoyl-ACP reductase (19) or of a β-hydroxyacyl-ACP dehydrase (14). Multiple species of both of these enzymes have been described (14, 19). Definition of the specific enzymatic defect in fabB is under investigation.

The number of recombinants formed in the transductional crosses indicates that fabA and fabB are not closely linked to each other although the members of a complementation group appear linked. The genetic mapping of these strains is in progress and will be the subject of a future communication.

ACKNOWLEDGMENTS

We are indebted to D. L. Wulff and C. Fred Fox for bacterial strains. We thank D. F. Silbert and D. L. Wulff for their valuable advice. J. E. Cronan was supported by a Public Health Service postdoctoral fellowship (1 F02 GM34773-01) from the National Institute of General Medical Sciences. C. H. Birge was supported by a Public Health Service predoctoral traineeship (5 T01 GM01311-04) from the National Institute of General Medical Sciences. This investigation was supported by U. S. Public Health Service grant 1-R01-HE 10406 and National Science Foundation grant GB-5142X.

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