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NOTES

The Presence of Linoleic Acid in *Escherichia coli* Cannot Be Confirmed

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***Escherichia coli* was recently reported to accumulate significant quantities of linoleic acid in stationary phase (H. Rabinowitch, D. D. Sklan, D. H. Chace, R. D. Stevens, and I. Fridovich, J. Bacteriol. 175:5324–5328, 1993). Since this finding would have considerable impact on the biochemical mechanisms of type II fatty acid synthases, we have attempted to confirm this observation. We found no evidence for the accumulation of linoleic acid in late-stationary-phase cultures of *E. coli* and conclude that the results of Rabinowitch et al. are artifactual.**

Recently, Rabinowitch et al. (9) reported that very late stationary phase cultures of *Escherichia coli* contain linoleic acid. Linoleic acid was reportedly present only in cultures older than 36 h, and the failure of prior investigators to detect these acids was attributed to analysis of younger cultures. This finding would have considerable impact on the understanding of lipid metabolism in *E. coli*, since the existence of linoleate suggests the presence of a hitherto unknown biochemical pathway for the anaerobic synthesis of polyunsaturated fatty acids by type II fatty acid synthase systems. However, we found this report unconvincing for the following reasons. (i) Previous investigators had examined cultures of several strains of *E. coli* grown for longer than 36 h and had not detected the presence of dienoic fatty acids (1, 3, 5, 6). For example, Law et al. (6) analyzed cultures periodically sampled during 122 h of growth and did not mention the detection of linoleic acid. Furthermore, two of these reports (1, 5) contained primary data in the form of gas-liquid chromatography tracings that did not give any indication of the presence of linoleate. (ii) The data of Rabinowitch et al. (9) are internally inconsistent, and several unexplained, aberrant chromatographic peaks in addition to the putative linoleate peak are also present. (iii) Rabinowitch et al. (9) failed to document the purity of the cultures analyzed, and the fatty acid compositions of their log-phase cultures are not typical of *E. coli.*

For these reasons, we have reinvestigated the claims of Rabinowitch et al. (9). The experiments were initiated independently in our two laboratories, and two complementary methods were used to assay for the presence of linoleate. In the Memphis laboratory, the parental strain analyzed by Rabinowitch et al. (9), strain AB1157 (*thr-1 leu-6 proA2 argE3 his-4 ara-14 galK2 lacY1 mtl-1 xyl-5 sup-37 tsx-33 rpsL*), was obtained from the Coli Genetic Stock Center and checked for its nutritional requirements. This strain was then grown for 24, 48, and 72 h at 37°C in minimal medium (0.4% glucose–0.001% thiamine–0.1% casein hydrolysate–100 μM pantothenate in M9 minimal salts) as described by Rabinowitch et al. (9). The culture reached stationary phase (1.5×10^9 cells per ml) at the 24-h time point, and the turbidity of the culture did not change after this time. At each time point, 25 ml of the culture was harvested, the lipids were extracted, and methyl esters were prepared with 3% HCl–methanol (2). Quantitation of the fatty acids present by gas-liquid chromatography using a flame ionization detector showed a typical *E. coli* fatty acid profile at all time points. The fatty acid profile at 72 h is shown in Fig. 1. In contrast to the report of 7.4% linoleate in strain AB1157 following 72 h of growth (9), we found no evidence for the presence of even trace quantities linoleic acid in our experiments.

In the Urbana laboratory, a different approach designed to avoid any possible chemical contamination was used. The isolate of strain AB1157 used in these experiments was obtained from J. Imlay of the University of Illinois, who transported the strain from the laboratory of Rabinowitch et al. (9). Fatty acids of strain AB1157 were labeled by growth in medium containing minimal M9 salts supplemented with 0.4% glucose plus 5 to 15 μCi of [U-^{14}C]glucose per ml and the required nutrients as recommended by Davis et al. (4). After inoculation (0.1 ml inoculum per 10 ml of radioactive medium) with a culture grown overnight in the same nonradioactive medium, the cultures were shaken at 37°C for 96 h, with samples taken and the lipids extracted at daily intervals. At the conclusion of the experiments, diluted samples of the cultures were plated on appropriately supplemented glucose-M9 agar plates to verify the sterility of the cultures and identify the strain. The cultured organisms required arginine, leucine, threonine, proline, and histidine as did strain AB1157. Moreover, the cells were resistant to streptomycin (as is strain AB1157) and sensitive to valine (a characteristic of *E. coli* K-12 strains) and formed colonies having a morphology characteristic of *E. coli* K-12. The culture samples (1.6 ml) were pipetted into chloroform-methanol (3 ml of a 1:2 [vol/vol] mixture) containing 10 μg of nonradioactive methyl linoleate (Nu-Check Prep Laboratories), and the lipids were extracted after

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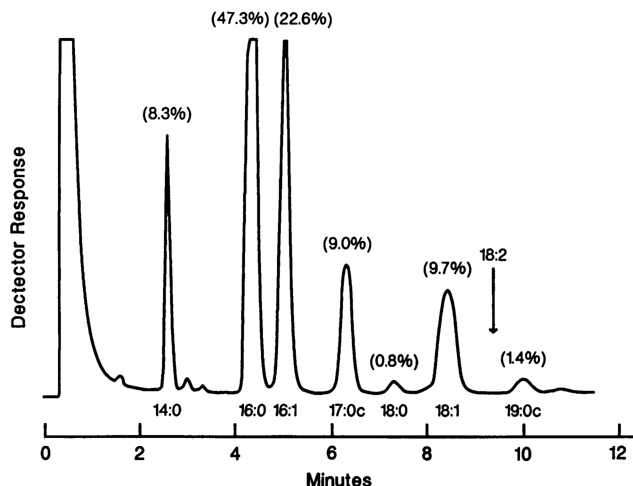

FIG. 1. Fatty acid composition of strain AB1157 grown in late stationary phase. Strain AB1157 was grown for 72 h in minimal medium, the lipids were extracted, and methyl esters were prepared as described in the text. The fatty acid methyl esters were analyzed by gas-liquid chromatography using a Hewlett-Packard 5890A instrument equipped with a flame ionization detector and a column packed with Chromsorb WAW (100/120 mesh) coated with 10% SP-2330 and operated isothermally at 190°C. Peaks were identified by comparison of their retention times with standards, and the integration accuracy was checked by using NHI standard mixture D. The indicated elution position of methyl linoleate was determined by chromatography of an individual standard and by spiking the *E. coli* fatty methyl ester mixture with methyl linoleate. The identity of each fatty acid is indicated as the number of carbon atoms:number of double bonds, and 17:0c and 19:0c refer to the cyclopropane derivatives of 16:1 and 18:1, respectively. The percentages shown are the weight percent of the total fatty acids detected.

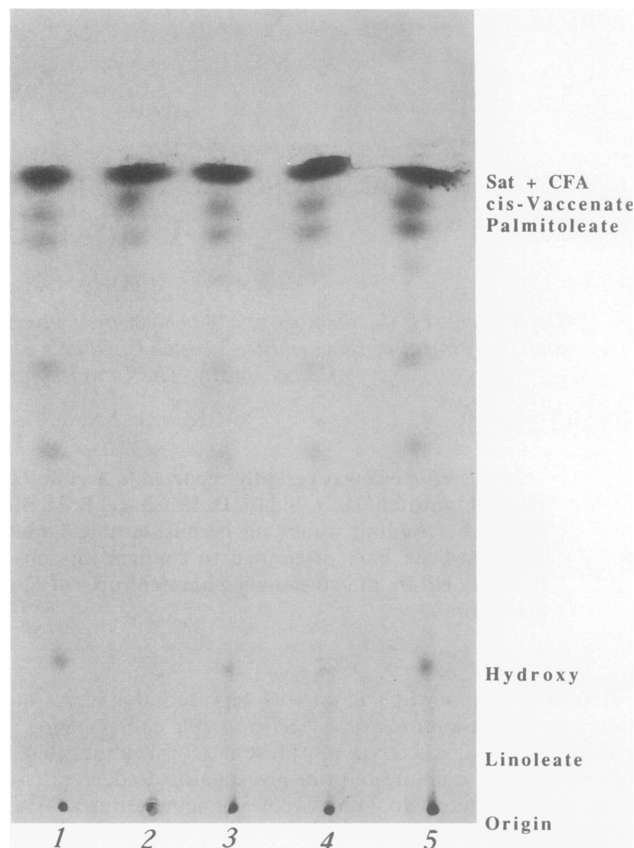

FIG. 2. Argentation thin-layer chromatography of the fatty acids derived from strain AB1157. A Silica Gel G plate was impregnated with 20% silver nitrate and activated as previously described (8). Samples containing the methyl esters of ^{14}C-labeled *E. coli* fatty acids plus 10 μg of methyl linoleate were applied to the plate in diethyl ether-hexane (1:3 [vol/vol]), and the plate was developed three times with toluene as described by Morris et al. (8). The plate was dried, autoradiographed for 6 days, and then sprayed with dichlorofluoroscein to detect the methyl linoleate internal standard (2). Samples were taken from a culture of strain AB1157 at the indicated times and then extracted as described in the text. Lane 1, 24 h; lane 2, 60.5 h; lane 3, 72 h; lane 4, 87.5 h; lane 5, 96 h. The hydroxy fatty acids were derived from lipid A present in outer membrane particles that contaminated the lipid extract. The minor labeled components migrating between the hydroxy and unsaturated esters are the isomeric tetradecenoates resulting from the dehydration of the lipid A 3-hydroxymyristate during methyl ester preparation (11). Sat + CFA, saturates and cyclopropane fatty acids.

the removal of insoluble material by low-speed centrifugation. Methyl esters were formed by transesterification catalyzed by either acid (3% HCl in methanol) or base (0.5 M sodium hydroxide in methanol) (2). The methyl esters were extracted and applied to Silica Gel G thin layers impregnated with silver nitrate (Fig. 2). This chromatographic system separates fatty acids by the number and location of their double bonds (2, 7). The greater the number of double bonds, the slower the migration due to increased liganding between the silver ions and the π orbitals of the double bonds (2, 8). Thus, methyl linoleate is easily separated from the mixture of saturates and monoenes found in *E. coli*. Cyclopropane species migrate with the saturated fatty acid methyl esters. Following development of the plate, autoradiography, and visualization, the appropriate areas were scraped from the plate, suspended in scintillant, and counted. No detectable radioactivity (<0.1% of the total radioactive fatty acids) was associated with the area of the plate containing methyl linoleate. These results were confirmed by two dimensional thin-layer chromatography in which the first dimension was chromatography on Silica Gel layers lacking silver nitrate and the second dimension was argentation chromatography. In these analyses, methyl linoleate and the methyl esters of *E. coli* fatty acids comigrate in the first dimension and are then separated according to the number of double bonds in the second dimension. No labeled linoleate was detected by this second method (not shown).

In summary, we have been unable to detect linoleic acid in late-stationary-phase cultures of *E. coli*. Our results are in agreement with the older literature (1, 3, 5, 6) and in direct conflict with the claim of Rabinowitch et al. (9). We believe that the results of Rabinowitch et al. (9) are artifactual. Their data might be explained by contamination of their cultures with a slowly growing eucaryotic microbe that synthesizes linoleate or a closely related diene. Rabinowitch et al. (9) failed to establish the purity of their late-stationary-phase cultures, and the presence of a slowly growing microbe could explain both the presence of linoleic acid (or a similar diene) and the delay in its appearance. Many common fungi and yeasts contain linoleic acid (8, 10). However, the very unusual fatty acid composition reported by Rabinowitch et al. (9) is also consistent with chemical contamination.

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