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Escherichia coli Produces Linoleic Acid during Late Stationary Phase

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***Escherichia coli* produces linoleic acid in the late stationary phase. This was the case whether the cultures were grown aerobically or anaerobically on a supplemented glucose-salts medium. The linoleic acid was detected by thin-layer chromatography and was measured as the methyl ester by gas chromatography. The linoleic acid methyl ester was identified by its mass spectrum. Lipids extracted from late-stationary-phase cells generated thiobarbituric acid-reactive carbonyl products when incubated with a free radical initiator. In contrast, extracts from log-phase or early-stationary-phase cells failed to do so, in accordance with the presence of polyunsaturated fatty acid only in the stationary-phase cells.**

Escherichia coli is known to contain saturated and monoenoic fatty acids during log-phase growth (18) and to accumulate cyclopropane fatty acids in the late log and early stationary phases (4). The function of the latter modification is unknown, since mutants defective in the cyclopropane fatty acid synthase (9) did not exhibit detectable phenotypic deficits (9, 10, 21). *E. coli* has not previously been found to contain endogenously produced polyunsaturated fatty acids (PUFA).

When nutrients become limiting or when very high cell densities have been achieved, *E. coli* stops growing and can survive in this apparently resting state (3, 6). Entry into this stationary phase is accompanied by changes in size, shape, and composition which, presumably, foster long-term survival (5, 19). We now report that linoleic acid biosynthesis is one of the metabolic changes which occur during stationary phase.

MATERIALS AND METHODS

Bacterial strains. Strains AB1157 (parental strain), JI130 [AB1157 (*sodA*::MudPR13)25] and JI132 (JI130 *sodB-kan*) (12, 13) were used.

Reagents. 1,1,3,3-Tetramethoxypropane, 2-thiobarbituric acid, pantothenic acid, and L-amino acids except L-serine were from Sigma; L-serine was from Fisher Biotech; chloroform and pyridine were from Fisher Scientific; sodium dodecyl sulfate was from BDH; sodium citrate, sodium acid phosphate, and catechol were from Matheson, Coleman and Bell; acetic acid and dibasic sodium phosphate were from Baker; 2,2′-azobis(2-amidino propane)-HCl (AAHP) was from Polyscience Inc., Warrington, Pa.; linoleic acid was from Serdary Research Labs Inc., London, Ontario, Canada. Silica gel-coated thin-layer chromatography (TLC) plates were 6060 silica gel (Eastman Kodak Co., Rochester, N.Y.), Polygram Sil G (Machery-Nagel and Company, Doren, Germany), and silica gel IB (Parker-Flex, J. T. Baker Chemical Company, Phillipsburg, N.J.). All other compounds were reagent grade from commercial sources.

Growth of *E. coli*. Unless otherwise specified, cultures were grown in 100 ml of minimal A salts (15) enriched with (per liter) 3 mg of pantothenate, 5 mg of thiamine, 2 g of glucose, 1.0 mM $MgSO_4$, and 0.5 mM each of the 20 naturally occurring amino acids. Media were made up in tap water to provide trace elements. Incubations were in 250-ml Ehrlenmeyer flasks at 200 rpm in a water bath at 37°C. Anaerobic incubations were done in a Coy chamber under 85% N_2, 10% H_2, and 5% CO_2. Cells were harvested at 10,000 rpm for 15 min at 22°C to avoid cold shock, either resuspended in fresh medium or transferred to 1.5-ml tubes, with a few crystals of catechol added as an antioxidant, and dehydrated in a Vacu-Fuge prior to extraction. Catechol was not added when extracts were to be assayed for content of antioxidant.

Assays. Culture density was measured as A_{500}, assuming 3.07×10^7 cells per ml per 0.1 absorbance unit (2). Lipids were extracted from cells with methanol-chloroform (2:1) as described by Kates (14). Lipid samples were then saponified by refluxing for 30 min in 90% ethanol containing 10% KOH. After cooling, nonsaponifiable lipids were removed by extraction with petroleum ether. After acidification with HCl, fatty acids were extracted into petroleum ether. For gas chromatography (GC), after evaporation of the solvent under a stream of N_2, the fatty acids were methylated by 60 min of refluxing in methanol–3% H_2SO_4. The methyl esters were then extracted with petroleum ether after addition of water, concentrated under N_2, and finally injected into a column (2 m by 1/8 in. [ca. 0.32 cm]) of 20% DEGS on Chromosorb operated at 180°C on a Hewlett-Packard 5890A GC (Hewlett-Packard Co., Avondale, Pa.). Heptadecanoic acid, added prior to saponification, was used as an internal standard (20). Extracts intended for TLC were prepared according to Kates (14). Thiobarbituric acid-reactive substances (1) were assayed as described by Ohkawa et al. (17), using tetramethoxypropane as the calibrating standard.

GC-MS. The instruments used were a Carlo Erba 8000 series GC and a MD800 benchtop quadrupole mass spectrometer (MS), both from Fisons Instrument, Danvers, Mass. The GC used a 30-m DB-23 fused silica capillary column from J and W Scientific, Folsom, Calif. The inside diameter of this capillary was 0.25 mm, and its film thickness was 0.25 μm. Splitless injection of 1 μl was followed after 45

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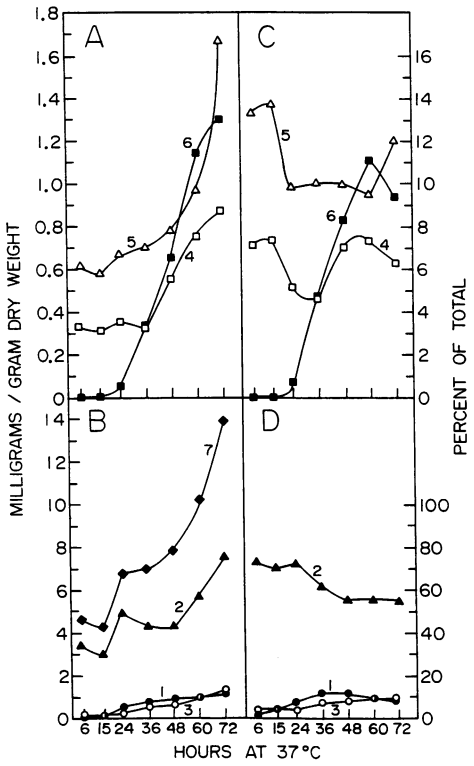

FIG. 1. Fatty acid composition as a function of culture age. *E. coli* AB1157 was cultured as described in Materials and Methods; at intervals, cells were harvested and extracted, and the methylated extracts were analyzed by GC. Panels A and B present the results in terms of milligrams of specific fatty acids per gram (dry weight) of cells; panels C and D give the amount of each fatty acid as a percentage of the fatty acids assayed. Fatty acids: line 1, 14:0; line 2, 16:0; line 3, 18:0; line 4, 16:1; line 5, 18:1; line 6, 18:2; line 7, total.

s by opening of the split. The injector temperature was 240°C. The oven temperature started at 80°C and was raised at 8°C/min to 275°C, where it was held for 5 min. The helium forepressure was 10 lb/in^2. The GC-MS interface temperature was 250°C. The electron energy was 70 eV, and the electron source temperature was 250°C. The mass range of 50 to 500 was scanned in 0.9 s, and there was a 0.1-s delay between scans.

The mixture of fatty acid methyl esters used as standards was obtained from Alltech Associates, Deerfield, Ill., and contained the methyl esters of hexanoate, heptanoate, octanoate, decanoate, dodecanoate, tetradecanoate, hexadecanoate, *cis*-9-hexadecenoate, octadecanoate, *cis*-9-octadecenoate, *cis*-9,12-octadecedienoate, *cis*-9,12,15-octadecetrienoate, and *cis*-5,8,11,14-eicosatetraenoate. Dried fatty acid samples from *E. coli* (~2 mg) were methylated prior to analysis by heating to 65°C for 20 min in 250 μl of 3.0 N HCl in methanol and then drying in vacuo. The resultant methyl esters were taken up in ethyl acetate.

RESULTS

E. coli grown in a glucose-salts medium supplemented with amino acids, thiamine, and pantothenate was harvested at intervals for extraction and analysis of fatty acids. The results in Fig. 1 show that total fatty acid content increased in stationary phase, very likely a reflection of the smaller size (5) of nongrowing cells and the consequent increase in the proportion of cell mass devoted to membranes. In agreement with previous workers (7, 16), we noted that palmitic acid (16:0) was dominant and that stearic acid (18:0) became more prominent in older cultures. Monounsaturated fatty acids (16:1 and 18:1) were the only unsaturated fatty acids found in log-phase cultures. However, as the cultures aged, a doubly unsaturated fatty acid (18:2) accumulated. The parental strain was compared with a *sodA* strain, JI130, and with a *sodA sodB* strain, JI132. All contained 18:2 fatty acid after 48 to 72 h of incubation, although the JI132 contained substantially less 18:2 fatty acid than did the superoxide dismutase-competent strains. This may be due to the slower aerobic growth of the JI132 (*sodA sodB*) strain, even in the amino acid-supplemented medium (12). At a given elapsed time, it was thus not as far into stationary phase as were the faster-growing superoxide dismutase-competent strains. These results are shown in Table 1, which also indicates that growth of the parental strain in the absence of oxygen did not significantly affect its content of 18:2 fatty acid after 72 h of incubation.

TABLE 1. Partial fatty acid content of *E. coli* AB1157, JI130, and JI132 grown in minimal medium A for 24 or 72 h

Strain	Air	Fatty acid (% of samples assayed)[a]			Total (mg/g [dry wt])
		Saturated	18:1	18:2	
24-h-old cultures					
JI132	+	91.3	8.7	0	2.18
JI130	+	86.9	13.1	0	4.52
AB1157	+	88.1	11.9	0	4.44
72-h-old cultures					
JI132	+	86.8	12.5	0.7	4.17
JI130	+	76.9	15.0	8.1	11.92
AB1157	+	79.6	13.0	7.4	13.93
AB1157	−	81.0	11.9	7.1	13.86

[a] Coefficient of variation was less than 15% in all cases (n = 3).

If PUFA were indeed present in the old but not the young cultures, then only the lipids extracted from the former should be susceptible to peroxidation. Methanol-chloroform extracts were prepared (14), saponified, dried under N_2, dissolved in 50 to 200 μl of $CHCl_3$, and converted into micelles by shaking vigorously with water. These suspensions were treated with the free-radical initiator AAPH added to 10 mM at 37°C. After 5 min of incubation with shaking, the accumulation of carbonyl products was assayed by the thiobarbituric acid test. Tetramethoxypropane was used as a standard source of malondialdehyde; the results are shown in Table 2. It is clear that susceptibility to lipid peroxidation appeared only in the stationary phase.

The possibility that extracts of *E. coli* might also contain an antioxidant was explored by measuring the oxidation of linoleic acid in the absence and in the presence of the chloroform-methanol extracts of the cells. Table 2 shows that the extract of 72-h cells strongly inhibited the oxidation of linoleic acid, whereas the extract of 6-h cells did not. The stationary-phase cells evidently contain a hydrophobic antioxidant which was not present in log-phase cells.

Since the production of PUFA by *E. coli* was such an unexpected finding, TLC with iodine vapor visualization was used as qualitative means of verification. Cultures were sampled at intervals, and extracts were prepared as described by Kates (14) and subjected to TLC on a variety of silica gel-coated plates along with a standard sample of

TABLE 2. Lipid peroxidation and antioxidant action in lipoidal extracts of *E. coli* as a function of culture age[a]

Assay	Culture age (h)	Reaction mixture	Malondialdehyde (nmol/10^9 cells)
Thiobarbituric acid test	6		<1
	12		<1
	24		<1
	36		2.3 ± 0.3
	72		4.9 ± 0.4
Oxidation of linoleic acid		Linoleic acid	17.0 ± 2.0
		72-h cell extract	4.1 ± 0.4
		Linoleic acid + 72-h extract	7.0 ± 0.7
		Linoleic acid + 6-h extract	17.0 ± 2.0

[a] AAPH was added to a final concentration of 10 mM, and incubations were carried out for 5 min at 37°C prior to analysis for carbonyl products with thiobarbituric acid. When the oxidation of linoleic acid was examined, its concentration was 12 mM.

linoleic acid. Linoleic acid was absent from 8-h cultures of AB1157 and became progressively more noticeable in 36-, 48-, and 72-h cultures of all three strains. Three different commercially available TLC plates were tested and found to give different migrations of linoleic acid relative to the solvent front (hexanes [boiling point, 65 to 70°C]-ethyl ether-acetic acid at 90:10:1, vol/vol/vol [14]). In each case, however, the fatty acid which appeared in the aged cultures comigrated with the authentic 18:2 fatty acid. These R_f values for authentic linoleic acid and for the 18:2 fatty acid in cell extracts were as follows: 6060 silica gel, 0.39; Polygram Sil G, 0.04; and silica gel IB, 0.06.

Cyclopropane fatty acids were estimated to constitute about 10% of the total fatty acids in the extracts of 72-h cells (data not shown).

Analysis of lipoidal extracts by GC-MS. Extracts were methylated, and the resultant methyl esters were analyzed as described in Materials and Methods. Figure 2 compares the GC-MS chromatograms of a mixture of fatty acid standards (Fig. 2A) with the patterns yielded by extracts of log-phase (Fig. 2B) and late-stationary-phase (Fig. 2C) cells. The late-stationary-phase cells clearly contained a prominent chromatographic peak corresponding with 18:2 fatty acid, whereas the log-phase cells did not. The peak at ~20 min in Fig. 2C indicates the presence of another compound in the extracts of the 72-h cells. This peak did not coincide with any of the standards run in Fig. 2A, and it remains unidentified.

The peak at 16.2 min was positively identified as linoleate by comparing its mass spectrum with that of a linoleate standard (Fig. 3). Figure 3A is the mass spectrum of the methyl ester of a *cis*-9,12-linoleic acid standard, while Fig. 3B is the corresponding mass spectrum of the chromatographic peak from the extract of late-stationary-phase cells which exhibited the same retention time as did the linoleate standard. The identities of the masses of the parent ions and of the many fragments generated therefrom are clear.

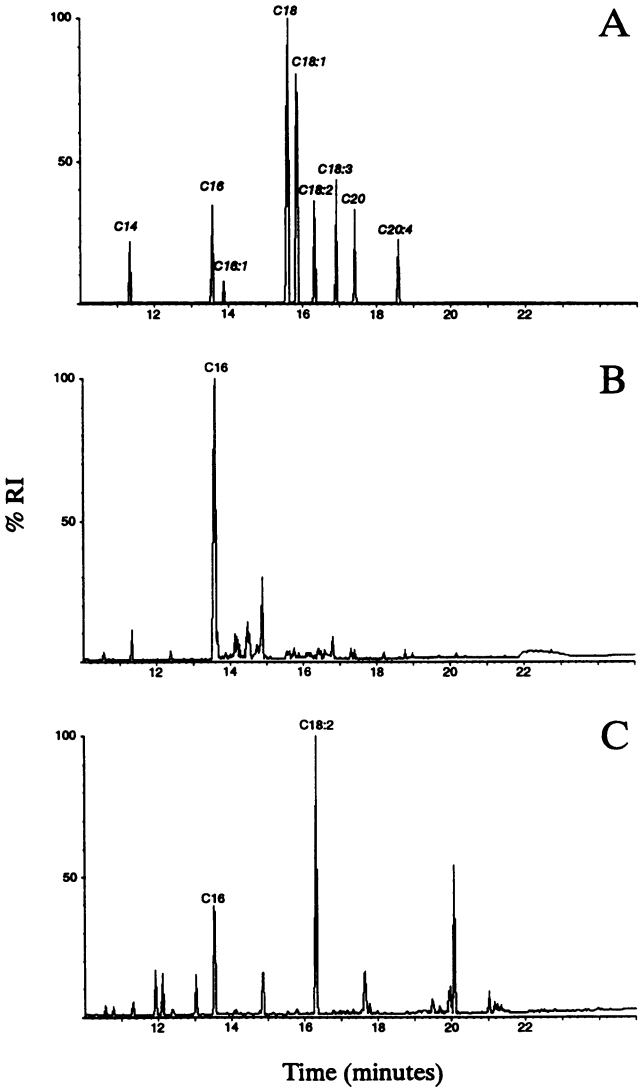

FIG. 2. GC-MS chromatograms of fatty acids from *E. coli*. (A) Mixture of methyl esters of fatty acid standards; (B) methyl esters of fatty acids prepared from the lipoidal extracts of log-phase cells; (C) same as panel B but from late-stationary-phase cells.

DISCUSSION

Except for cases in which PUFA were supplied in the medium to fatty acid auxotrophs (7, 11), there have been no previous reports of methylene-interrupted PUFA in *E. coli*. Given the appearance of 18:2 fatty acid only in late stationary phase, we must suppose that its biosynthesis is another example of the multiple adaptations which *E. coli* makes under these conditions (6, 19). It is worth recalling that this process occurred both aerobically and anaerobically. This finding is of interest since the known fatty acid desaturases act by an O_2-dependent mechanism. It is also worth noting

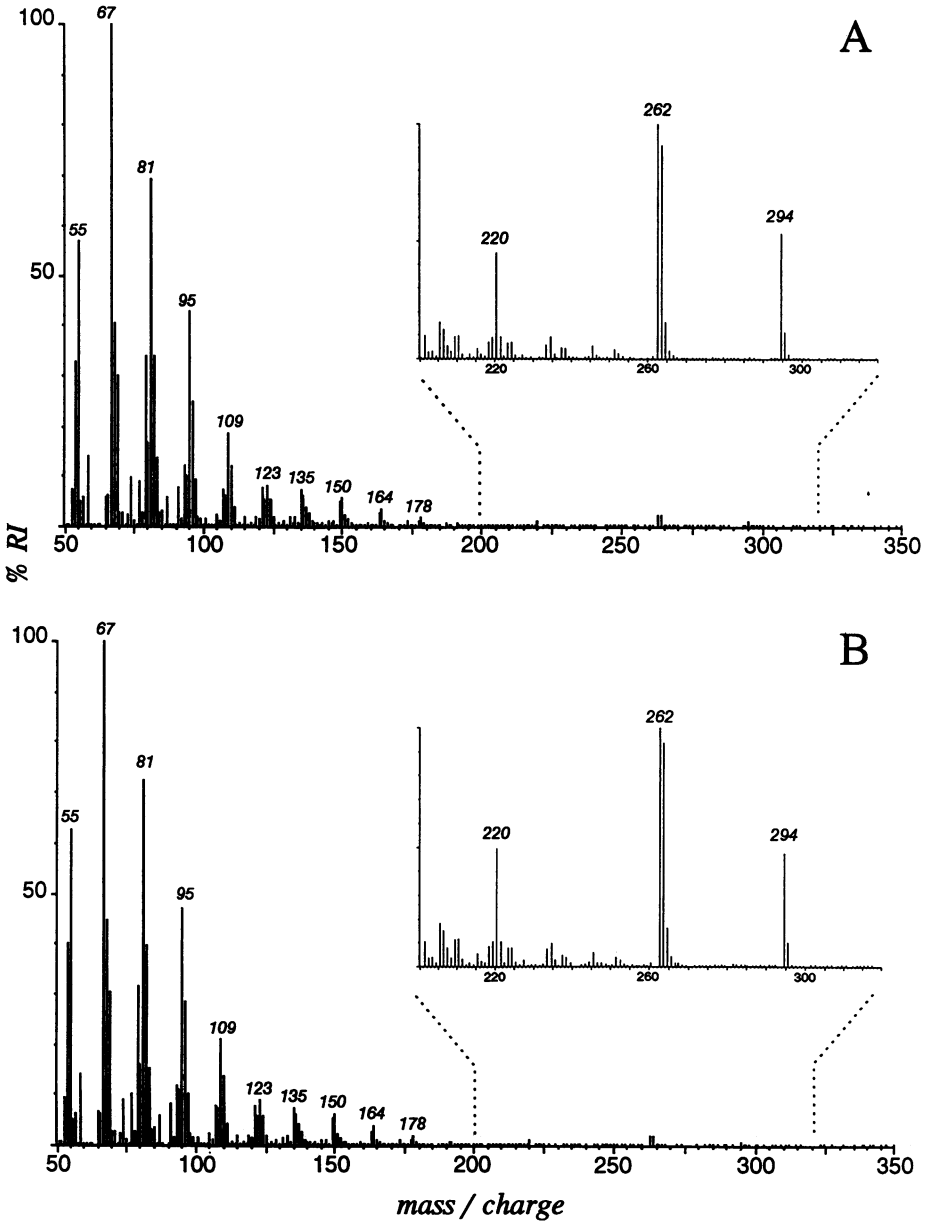

FIG. 3. Identification of the component from stationary-phase cells whose retention time was 16.3 min. (A) Mass spectrum of methyl linoleate; (B) mass spectrum of the 16.3-min peak in the gas chromatogram of the material from the late-stationary-phase culture.

that we were able to use an inoculum taken from a 72-h culture and replicate the observation by GC of no 18:2 fatty acid in log-phase cells grown from that inoculum. This finding establishes that mutation and selective outgrowth of an 18:2 fatty acid-producing mutant were not responsible for the appearance of 18:2 fatty acid in the late-stationary-phase cultures.

It will now be of interest to examine the metabolic pathways by which fatty acid desaturation occurs in *E. coli* under both aerobic and anaerobic conditions and the survival advantages provided by PUFA to stationary-phase cells. The identity of the hydrophobic antioxidant(s) and its metabolism will also require exploration.

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