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Mechanism of Biosynthesis of Unsaturated Fatty Acids in *Pseudomonas* sp. Strain E-3, a Psychrotrophic Bacterium

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Biosynthesis of palmitic, palmitoleic, and *cis*-vaccenic acids in *Pseudomonas* sp. strain E-3 was investigated with in vitro and in vivo systems. [1-^{14}C]palmitic acid was aerobically converted to palmitoleate and *cis*-vaccenate, and the radioactivities on their carboxyl carbons were 100 and 43%, respectively, of the total radioactivity in the fatty acids. Palmitoyl coenzyme A desaturase activity was found in the membrane fraction. [1-^{14}C]stearic acid was converted to octadecenoate and C_{16} fatty acids. The octadecenoate contained oleate and *cis*-vaccenate, but only oleate was produced in the presence of cerulenin. [1-^{14}C]lauric acid was aerobically converted to palmitate, palmitoleate, and *cis*-vaccenate. Under anaerobic conditions, palmitate (62%), palmitoleate (4%), and *cis*-vaccenate (34%) were produced from [1-^{14}C]acetic acid, while they amounted to 48, 39, and 14%, respectively, under aerobic conditions. In these incorporation experiments, 3 to 19% of the added radioactivity was detected in released $^{14}CO_2$, indicating that part of the added fatty acids were oxidatively decomposed. Partially purified fatty acid synthetase produced saturated and unsaturated fatty acids with chain lengths of C_{10} to C_{18}. These results indicated that both aerobic and anaerobic mechanisms for the synthesis of unsaturated fatty acid are operating in this bacterium.

Temperature-dependent changes in the ratio of unsaturated to saturated fatty acids in membrane lipids have been considered to be one of the regulatory mechanisms that maintain membrane fluidity (17). Thus, it is of much interest to study the factors affecting the ratio of unsaturated to saturated fatty acids. Since most but not all bacteria (9) contain only monounsaturated fatty acids as the unsaturated ones, it is convenient to study thermal regulation of the fatty acid composition of the membrane lipids.

Fatty acid synthetases of many organisms including animals, plants, fungi, and some bacteria produce exclusively saturated fatty acids (10). In these organisms, unsaturated fatty acids are synthesized by oxygen-dependent fatty acid desaturase systems (aerobic mechanism [12]). On the other hand, some bacteria such as *Escherichia coli* (4), *Clostridium butyricum* (11), and *Brevibacterium ammoniagenes* (14) synthesize monounsaturated fatty acids by fatty acid synthetases in an oxygen-independent manner (anaerobic mechanism). Scheuerbrandt and Bloch (19) reported that *Pseudomonas fluorescens*, a strict aerobe, also synthesizes monounsaturated fatty acids by the anaerobic mechanism. Since then, the bacteria belonging to the genus *Pseudomonas* have been referred to as representatives having the anaerobic mechanism (8, 10).

Recently, we (20) indicated that *Pseudomonas* sp. strain E-3, a psychrotrophic bacterium, has a fatty acid desaturase system which catalyzes the aerobic production of palmitoleate from palmitate. To examine the biosynthetic pathway of unsaturated fatty acids in this bacterium, we analyzed the fatty acids produced by in vivo and in vitro systems.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Psychrotrophic bacterium *Pseudomonas* sp. strain E-3 (*Pseudomonas* strain E-3) was isolated by us from dust and dirt in a cold room (gram negative, strict aerobe, glucose nonfermentative, oxidase and catalase positive [18]). *Pseudomonas* strain E-3 was cultivated in a Tris-salt medium with succinate as the sole carbon source with shaking (20) at 15°C for 24 h (mid-logarithmic phase).

Incorporation of radioactive fatty acids. A total of 1 μCi of [1-^{14}C]palmitic acid, [1-^{14}C]stearic acid, or [1-^{14}C]lauric acid was added to 100 ml of the culture. For analysis of de novo synthesis of fatty acid, 0.3 μCi of [1-^{14}C]acetic acid was added to 100 ml of the culture. The cultures were incubated for a further 1 h at 15°C with shaking. Then the cultures were chilled in ice water, and the cells were harvested and washed with chilled deionized water by centrifugation at 3,000 × *g* for 10 min at 4°C. For the determination of released $^{14}CO_2$, 50 ml of air per min flowed through the culture and was bubbled into 5 ml of Hyamine 10X. After the incubation, 0.3 ml of Hyamine 10X and 5 ml of toluene-based scintillation cocktail [2,5-diphenyloxazole, 4 g; 2,2′-*p*-phenylenebis(5-phenyloxazole), 50 mg; per liter of toluene] were mixed in a scintillation vial, and the radioactivity was counted with a liquid scintillation counter. For the experiment under anaerobic conditions, dissolved oxygen was removed and the atmosphere of the culture was filled with N_2 gas (O_2, less than 5×10^{-5}%) as previously described (20).

Lipid extraction and fractionation. Total lipids were extracted from the whole cells by the method of Bligh and Dyer (3). Total lipids were methanolyzed in 5% methanolic HCl by heating at 90°C for 3 h. Resulting methyl esters were extracted three times with hexane and concentrated under a stream of N_2 gas. Radioactive fatty acids were analyzed by radio gas-liquid chromatography and silver nitrate thin-layer chromatography.

Oxidative cleavage of palmitoleate methyl ester was performed by permanganate-periodate oxidation (1). Resulting monocarboxylic and dicarboxylic acid fragments were transmethylated with diazomethane and analyzed by radio gas-liquid chromatography.

Determination of carboxyl carbon labeling of fatty acids. Methyl esters of palmitic, palmitoleic, and *cis*-vaccenic acids were separated by consecutive chromatography on reverse-phase and silver nitrate thin-layer plates as described previ-

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ously (20). Each extract of isolated methyl esters was quantitatively poured into a scintillation vial (for control) and a Warburg flask and evaporated to dryness. The carboxyl carbon of fatty acids was released as CO_2 by the method of Brady et al. (6) with a minor modification. Before the decarboxylation reaction, a minivial (volume, 0.5 ml) was put on the center well of the Warburg flask. The Warburg flask was capped with a rubber stopper and tightly sealed with vinyl tape and rubber bands to withstand a rising inner pressure when heated. After the decarboxylation reaction took place at 70°C for 1 h, the Warburg flask was cooled in ice-water. Then 0.3 ml of Hyamine 10X was injected into the minivial with a hypodermic syringe through the rubber stopper. After absorption of CO_2 with Hyamine 10X, the minivial was transferred into a scintillation vial. To this vial was added 5 ml of toluene-based scintillation cocktail, and the radioactivity was measured with a liquid scintillation counter. The control value of radioactivity was determined with nondecarboxylated fatty acid methyl esters. The percentage of carboxyl carbon labeling of fatty acids was calculated from the above two values.

Preparation of in vitro fatty acid-synthesizing system. Cells were sonically disrupted in 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-mercaptoethanol. Unbroken cells and cell debris were removed by centrifugation at 10,000 × *g* for 20 min. The supernatant was centrifuged again at 100,000 × *g* for 90 min. The supernatant of the 100,000 × *g* centrifugation was fractionated with 55 to 75% saturated ammonium sulfate and then treated with activated charcoal. All the above procedures were done at 4°C. Acyl carrier protein (ACP) was prepared from *E. coli* cells by the method of Rock and Cronan (16). ACP from *Pseudomonas* strain E-3 was also prepared from the supernatant remaining after 75% saturated ammonium sulfate fractionation. The supernatant was acidified to pH 3.9 with added glacial acetic acid and stood overnight at room temperature. The precipitate was collected by centrifugation (8,000 × *g*, 10 min) and suspended in an appropriate amount of 50 mM sodium phosphate buffer (pH 7.0). The precipitate was dissolved by neutralization with 1 M Tris base. The solution thus obtained was used as *Pseudomonas* strain E-3 ACP.

Protein concentration was estimated by the method of Bradford (5).

Preparation of membrane fraction. Membrane fraction was prepared from cells suspended in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.25 M sucrose. Cells were sonically disrupted, and unbroken cells and cell debris were removed by centrifugation at 10,000 × *g* for 20 min. The supernatant was centrifuged again at 100,000 × *g* for 90 min, and the precipitate was suspended in the same buffer to give a protein concentration of approximately 2 mg/ml. This fraction was used as the membrane fraction. All procedures were done at 4°C. Desaturase activity was assayed within the same day of membrane preparation.

Enzyme assay. (i) In vitro fatty acid-synthesizing system. The reaction mixture (total volume, 500 μl) contained 50 μM acetyl coenzyme A (CoA), 20 μM [2-^{14}C]malonyl-CoA, 900 μM NADPH, 900 μM NADH, 40 μg of ACP, 140 μg of enzyme protein, and 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-mercaptoethanol. The reaction was initiated by the addition of enzyme protein, performed for 1 h at 15°C, and terminated by the addition of 10% methanolic KOH. The reaction mixture was saponified at 80°C for 30 min. After acidification of the mixture to pH 3.0 with 5 N H_2SO_4, free fatty acids were extracted with diethyl ether. The procedure for conversion of fatty acids to methyl esters was the same as described above except that 1 mg of unlabeled total lipids from *Pseudomonas* strain E-3 was added as carrier lipids. Distribution of radioactivity was examined by radio gas-liquid chromatography. For the experiment under anaerobic conditions, the reaction mixture was continuously bubbled with N_2 gas (O_2, less than 5×10^{-5}%) during the incubation.

(ii) In vitro fatty acid desaturase. The reaction components were as follows: 0.1 M sodium phosphate buffer (pH 7.0), 100 μM NADPH, 100 μM [1-^{14}C]palmitoyl-CoA (27 mCi/mol) or [1-^{14}C]stearoyl-CoA (26 mCi/mol), and 350 μg of membrane protein in a total volume of 500 μl. To equilibrate the temperature, the membrane fraction and the mixture of the other components were separately preincubated at 25°C for 2 min. The reaction was initiated by mixing all the components and carried out at 25°C for 4 min. The reactions were terminated by adding 500 μl of 10% methanolic KOH. The procedures for the saponification, acidification, extraction into diethyl ether, and conversion of fatty acids to methyl esters were the same as described for the in vitro fatty acid-synthesizing system. For the separation of saturated and unsaturated fatty acids, their methyl esters were chromatographed on silver nitrate thin-layer plates as described previously (20). The radioactivity was counted with a liquid scintillation counter (20). Experimental procedures for anaerobic conditions were the same as described above.

Preparation of [1-^{14}C]palmitoyl-CoA and [1-^{14}C]stearoyl-CoA. [1-^{14}C]palmitoyl-CoA was synthesized from CoA-SH and a mixture of [1-^{14}C]palmitic acid and unlabeled palmitic acid by the method of Bishop and Hajra (2). [1-^{14}C]stearoyl-CoA was prepared by the same method from CoA-SH, [1-^{14}C]stearic acid, and unlabeled stearic acid.

Chemicals. [1-^{14}C]palmitic acid (55.8 mCi/mmol), [1-^{14}C] stearic acid (60.0 mCi/mmol), [1-^{14}C]lauric acid (54.0 mCi/mmol), and [2-^{14}C]malonyl-CoA (55.8 mCi/mmol) were purchased from Amersham International, and the sodium salt of [1-^{14}C]acetic acid (15 mCi/mmol) was from ICN Radiochemicals. CoA was purchased from Kyowa Hakko Kogyo Co. NADH, NADPH, and ATP were products of Oriental Yeast Co. Hyamine 10X was obtained from Nakarai Chemicals Ltd. Cerulenin was purchased from Sigma Chemical Co. (St. Louis, Mo.). All other reagents used were of analytical grade.

RESULTS

Incorporation of radioactive fatty acids with whole-cell system. When [1-^{14}C]palmitic acid was added to a mid-logarithmic-phase cell culture, a considerable amount of the added radioactivity was incorporated into cellular lipids (chloroform-extractable fraction) and concomitantly released as $^{14}CO_2$. Radioactivity was incorporated into palmitate, hexadecenoate, and octadecenoate (Table 1). These unsaturated fatty acids were identified as palmitoleate and *cis*-vaccenate, respectively, by silver nitrate-impregnated thin-layer chromatography as reported previously (20). When [1-^{14}C]stearic or [1-^{14}C]lauric acid was added to the cell culture, less radioactivity was recovered in cellular lipids and released $^{14}CO_2$ than from [1-^{14}C]palmitic acid; however, unsaturated fatty acids were labeled significantly under aerobic conditions. We found that radioactivity from [1-^{14}C]stearic or [1-^{14}C]lauric acid was incorporated into palmitoleate. Labeled octadecenoate from [1-^{14}C]stearate was identified as a mixture of oleate and *cis*-vaccenate (Fig. 1A); however, only *cis*-vaccenate was found from [1-^{14}C] laurate. When cerulenin was present in the reaction mixture

TABLE 1. Distribution of radioactivity among fatty acids in total lipids in *Pseudomonas* strain E-3

Labeled precursor	Atmospheric condition	Amt added to culture (nmol/ml)	% of added radioactivity in lipids	% of added radioactivity in $^{14}CO_2$	Distribution of radioactivity (% of total radioactivity)			
					16:0[a]	16:1	18:0	18:1
[1-^{14}C]acetic acid	Aerobic	0.20	27.0	18.6	48	39	0	14
	Anaerobic	167.00[b]	0.5		62	4	0	34
[1-^{14}C]lauric acid	Aerobic	0.19	1.2	2.7	36	33	0	30
	Anaerobic	0.19			97	3	0	0
[1-^{14}C]palmitic acid	Aerobic	0.18	20.0	14.0	80	18	0	2
[1-^{14}C]stearic acid	Aerobic	0.17	3.2	5.1	2	7	65	26

[a] Number of carbons:number of double bonds.
[b] To determine the composition of the radioactive fatty acids which were produced from [1-^{14}C]acetic acid under anaerobic conditions, we added 25 μCi (1.67 μmol) of the substrate to 10 ml of the culture to overcome low incorporation.

with [1-^{14}C]stearate, *cis*-vaccenate was not labeled (Fig. 1B). Under anaerobic conditions, radioactivity from [1-^{14}C]stearate was slightly incorporated (0.6% of added radioactivity) into cellular lipids, but any unsaturated fatty acid was not labeled. The composition of fatty acids synthesized de novo from [1-^{14}C]acetic acid was almost the same as that reported previously (20). Under anaerobic conditions, only a small amount of radioactivity from [1-^{14}C]acetate was incorporated into cellular lipids, but *cis*-vaccenate was significantly labeled.

We measured the percentage of carboxyl carbon labeling in radiolabeled fatty acids derived from [1-^{14}C]palmitic acid. The percentages of ^{14}C in the carboxyl carbon were 101.3 ± 3.1, 99.7 ± 6.3, and 42.5 ± 3.0 for palmitate, palmitolate, and *cis*-vaccenate, respectively [expressed as (radioactivity released as $^{14}CO_2$ by C-1 decarboxylation/total radioactivity of the fatty acid) × 100; average of three experiments ± standard deviation], showing that the carboxyl carbons of palmitate and palmitoleate were directly derived from the C-1 carbon of added [1-^{14}C]palmitate, whereas the radioactivity of the carboxyl carbon of *cis*-vaccenate was diluted with cold carbon. These results obtained from in vivo experiments indicated that palmitate and stearate are directly desaturated to palmitoleate and oleate, respectively. We analyzed the distribution of radioactivity in palmitoleate produced from [1-^{14}C]laurate. The radioactivity was distributed in the C_9 dicarboxylic acid of a permanganate-periodate-cleaved product which corresponds to C-1 to C-9 of palmitoleate (Fig. 2).

In vitro fatty acid desaturase system. To confirm the existence of the aerobic fatty acid desaturase system in *Pseudomonas* strain E-3, we examined desaturase activity in vitro. The membrane fraction, but not the soluble fraction, was able to desaturate palmitoyl-CoA (Table 2). The membrane fraction also desaturated stearoyl-CoA to oleate (Fig. 1C). Palmitoyl-CoA desaturase activity depended on molecular oxygen and NADPH or NADH (Table 2). Furthermore, the desaturase activity was completely inhibited by respiratory inhibitors such as NaN_3 and KCN and by the sulfhydryl inactivator mercuric chloride. These properties of the desaturase system are similar to those of acyl-CoA desaturase systems from other organisms (12).

In vitro fatty acid-synthesizing system. It has been thought that the fatty acid synthetases of organisms possessing the aerobic mechanism do not produce unsaturated fatty acids (10). We examined whether the fatty acid synthetase of *Pseudomonas* strain E-3 agreed with this hypothesis. Activ-

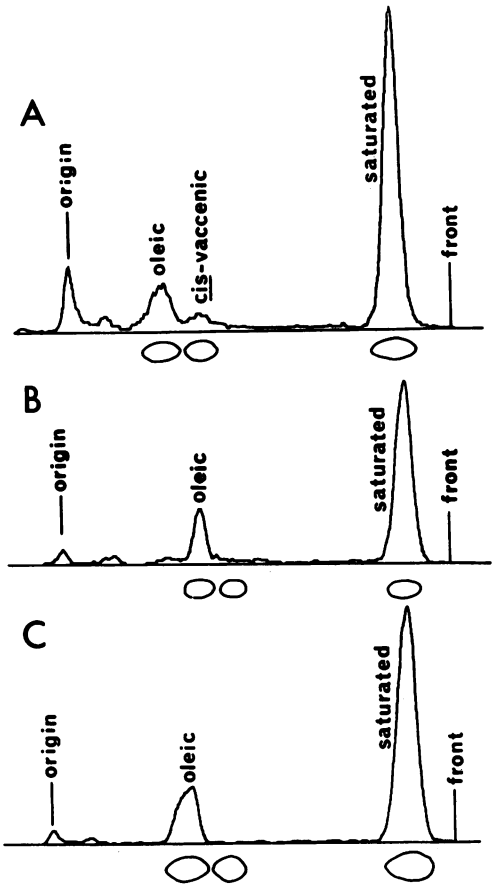

FIG. 1. Radioactivity tracings of thin-layer chromatograms of fatty acid methyl esters. Methyl esters were separated on 20% silver nitrate-impregnated silica plates (Kieselgel 60H) with toluene as the developing solvent with triple development (one-half, one-third, and top of plate) at −25°C. Radioactively labeled areas were located with a radioactive chromatogram analyzer. (A) [1-^{14}C]stearic acid was added to the cell culture and incubated at 15°C for 1 h. Total lipids were extracted, methanolyzed, and subjected to silver nitrate thin-layer chromatography. (B) Same as panel A except that incubation was carried out in the presence of cerulenin (100 μg/ml). (C) Products of in vitro [1-^{14}C]stearoyl-CoA desaturase. Incubation was carried out at 25°C for 15 min.

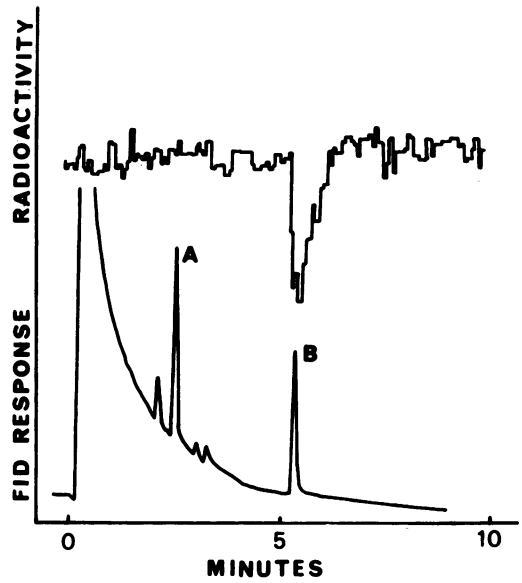

FIG. 2. Radio gas-liquid chromatogram of methyl esters of oxidation products from [^{14}C]palmitoleate which is produced from [1-^{14}C]lauric acid. Flame ionization detector (FID) response and radioactivity tracing are shown. The methyl esters were separated on a 3% Silicone SE-30 column (3 mm by 2 m) at an N_2 gas flow rate of 30 ml/min. The column temperature was programmed from 100 to 190°C at 10°C/min and maintained at 190°C. (A) C_7 monocarboxylic acid methyl ester; (B) C_9 dicarboxylic acid methyl ester.

ity of the partially purified fatty acid-synthesizing system of *Pseudomonas* strain E-3 depended on ACP (data not shown). Like the system of *E. coli* (15), the chain length of the fatty acids and the ratio of saturated to unsaturated fatty acids produced were varied by alteration of the assay conditions such as temperature and proportion in concentrations of acetyl-CoA and malonyl-CoA, NADPH and $NADP^+$, and NADH and NAD^+ (data not shown). However, both saturated and unsaturated fatty acids were always synthesized irrespective of the assay conditions (Table 3). In the assay system employing ACP from *Pseudomonas* strain E-3, both types of fatty acids were also produced (Table 3). A possibility that contaminated membrane fraction contributes to unsaturated fatty acid production was excluded by the finding that the composition of anaerobically produced fatty acids was the same as that produced aerobically (data not shown).

DISCUSSION

In bacteria, either an aerobic or an anaerobic mechanism is responsible for the biosynthesis of unsaturated fatty acids

TABLE 2. In vitro desaturase activity

System	Unsaturated fatty acid production (nmol/mg of protein per min)
[1-^{14}C]palmitoyl-CoA as substrate	
Complete system	0.84
− NADPH	0.07
− NADPH, + NADH	0.62
Boiled membrane	0
Soluble fraction	0
Nitrogen atmosphere	0
[1-^{14}C]stearoyl-CoA as substrate (complete system)	0.73

TABLE 3. Product distribution of fatty acid-synthesizing system

ACP source	Distribution of radioactivity in fatty acids (%)							U/S ratio[a]
	10:0[b]	12:0	14:0	16:0	16:1	18:0	18:1	
E. coli	4	2	16	28	24	15	11	0.54
Pseudomonas strain E-3	10	2	14	35	17	12	10	0.37

[a] Ratio of unsaturated to saturated fatty acids.
[b] Number of carbons:number of double bonds.

(10). Scheuerbrandt and Bloch (19) analyzed positions of double bonds in monounsaturated fatty acids of many microorganisms and showed that the microorganisms with the anaerobic mechanism contain a series of homologous even-numbered unsaturated fatty acids in which double bonds are located between carbon atoms 7 and 8 counting from the methyl end of the fatty acid molecule, i.e., Δ^7-C_{14}, Δ^9-C_{16}, and Δ^{11}-C_{18}. However, if a double bond is introduced between carbon atoms 7 and 8 by aerobic desaturation and then the chain is successively lengthened anaerobically with a C_2 unit as is done by cellular slime mold (7) and rat liver (13), the same set of monounsaturated fatty acids would be produced. Existence of the aerobic mechanism in *Pseudomonas* strain E-3 was demonstrated by in vivo and in vitro experiments with radiolabeled palmitate and stearate at the C-1 position.

Furthermore, production of radiolabeled unsaturated fatty acids from [1-^{14}C]laurate is additional evidence for the operation of the aerobic mechanism in this bacterium. If unsaturated fatty acids are anaerobically produced at the step of dehydration of 3-hydroxydecanoyl-ACP as reported with *E. coli* (4), palmitoleate and *cis*-vaccenate would not be produced, because elongation series of saturated and unsaturated fatty acids longer than C_{10} are distinct in the anaerobic mechanism (4).

On the other hand, the partially purified fatty acid-synthesizing system produced both saturated and unsaturated fatty acids in an oxygen-independent manner, and both fatty acids were also anaerobically produced from acetic acid with the in vivo system. Therefore, we conclude that both aerobic and anaerobic mechanisms for the biosynthesis of unsaturated fatty acids are able to function in this bacterium. Anaerobic production of unsaturated fatty acids in vivo seems to be insufficient, because nitrate did not support anaerobic growth of *Pseudomonas* strain E-3 (data not shown). However, it could not be determined at present to what extent the respective mechanisms were concerned with in vivo synthesis of unsaturated fatty acids under aerobic conditions, because added long-chain fatty acids were metabolized differently, for instance, by β-oxidation, aerobic desaturation, or anaerobic resynthesis of fatty acids from oxidatively decomposed products. Since we obtained evidence for the coexistence of aerobic and anaerobic mechanisms in *Pseudomonas aeruginosa* (data not shown), we presume the same mechanisms are widely distributed in many bacterial strains belonging to the genus *Pseudomonas*.

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