JOURNAL OF BACTERIOLOGY, Sept. 1976, p. 1389-1399
Vol. 127, No. 3

Printed in U.S.A.

# Septation Deficiency and Phospholipid Perturbation in *Escherichia coli* Genetically Constitutive for the Beta Oxidation Pathway

EDGARD VANDERWINKEL,* MONIQUE DE VLIEGHERE, MICHEL FONTAINE, DANIEL CHARLES, FRANÇOISE DENAMUR, DANIÈLE VANDEVOORDE, AND DANIEL DE KEGEL

*Research Institute and Laboratory of Applied Biochemistry IIF-IMC, Centre d'Etudes et de Recherches des Industries Alimentaires et Chimiques, B-1070 Brussels, Belgium,* and Laboratory of Electron Microscopy, Pasteur Institute of Brabant, B-1040 Brussels, Belgium*

Received for publication 4 May 1976

Mutants of *Escherichia coli* defective in the regulation of the fatty acid beta oxidation pathway show an ultrastructural deficiency in septum formation at high growth rate. Several independent pairs of parent and mutant strains have been analyzed biochemically. Each parent strain displays a well-defined pattern of cellular phospholipids, which varies with the growth conditions. High ratios of phosphatidylglycerol to cardiolipin characterize fast-growth conditions. None of the mutant strains, although they grow in mass nearly as rapidly as their respective parents, can reach these high ratios. The beta oxidation pathway regulatory mutation leads to an increased turnover of the glycerol moieties of these phospholipids in the inner as well as in the outer cell membrane.

Appropriate fixation procedures for electron microscopy of *Escherichia coli* have allowed the preservation of the highly labile structures occurring in cell division (5); in addition, timing of the septation events has been possible by the use of synchronous cultures (6).

Accomplishment of this sequence of events implies the regulated expression of several genes, some of which are known. Numerous mutants, defective in septation under nonpermissive conditions, have distinctive physiological behaviors, and the mutations have been mapped (1, 25). Some of them have alterations in phospholipid and/or murein synthesis (22). Three other nonconditional mutations (*envA,B,C*) leading to an impaired cell division due to membrane alteration have also been located (16, 26).

The biochemical links between the well-defined structural features and the physiological properties of these mutants remain poorly understood. Our attention was drawn, therefore, to the observation that a biochemically known mutation, the constitutivity of the fatty acid beta oxidation pathway, leads to a defect in the septation process: on rich medium, cells grow in a filamentous form. The regulation of fatty acid catabolism is well documented by the work of Overath et al. (15, 21). Like these authors, we selected and used constitutive mutants (32) for this catabolic pathway. The mutation designated FadR by Overath and OleR by us can be easily selected on a medium supplemented with butyrate, valerate (32), or decanoate (21) as the sole carbon source; preliminary results (not published) obtained in our laboratory indicate that the mutation presently designated as *oleR* is located near *purB* (at 25 min on chromosome map) with which it is 35% co-transducible by phage P1*kc*.

Results presented here on morphological structures and on biochemical analysis of several pairs of parent and mutant strains grown under three different conditions emphasize a possible leading role of phospholipids in the septation process.

## MATERIALS AND METHODS

**Strains.** All bacterial strains used were *Escherichia coli* K-12. The sources from which they were obtained and their properties are shown in Table 1.

Specific activities of the beta oxidation enzymes of constitutive strains were about the same after growth in a rich medium (no. 853) or in a glucose-salts medium; they were generally 20 times higher than the parental levels (32).

**Bacterial media and growth.** Mineral salts-glucose medium was the medium 132 described previously (31), with a final glucose concentration of 5 g/liter. Metabolite requirements were satisfied as follows (final concentrations): L-cysteine, L-histidine, and L-arginine, 100 μg/ml; thiamine, 1 μg/ml. Medium 853 contains (per liter): tryptone (Difco), 10 g; yeast extract (Difco), 5 g; NaCl, 5 g; glucose, 1 g; $K_2HPO_4$, 0.7 g; and $KH_2PO_4$, 0.3 g. The rich medium used for strain 8 (8) and its derivative 8-01 contains 10 g of Casamino Acids (vitamin and salt free)

TABLE 1. *Bacterial strains*

| Strains | Properties | Source/reference |
|---|---|---|
| RC-10 | F⁻ *cysC* Str$^r$ | R. Lavallé |
| RC10-9 | OleR derivative of RC-10 | Selected on decanoate |
| D5H3G7 | F⁻ *aceD his* Str$^r$ | 32 |
| TRF5 | OleR derivative of D5H3G7 | 32 |
| 37D52 | F⁻ *aceD his arg* Str$^r$ | Recombinant of PA3730 (R. Lavallé) |
| D52-81 | OleR derivative of 37D52 | Selected on decanoate |
| KL16 | Hfr | B. Low |
| KL16-72 | OleR derivative of KL16 | Selected on decanoate |
| 8 | Kfr Cavalli *glpD* GlpR | E. C. C. Lin |
| 8-01 | OleR derivative of strain 8 | Selected on decanoate |

(Difco) and 1 g of glucose per liter of mineral salt medium 132.

Cultures were grown at 37 or 30°C on a rotary shaking incubator, and turbidity measurements were performed at 660 nm with a Beckman B spectrophotometer.

Viable counts were determined by taking duplicate samples at the desired absorbance value and making serial dilutions in the respective media in parallel on each sample. Duplicate samples of the suitably diluted bacterial suspensions were spread immediately on nutrient agar plates, and the colonies were counted after 18 h of incubation.

**Optical and electron microscopy.** Eosin-Giemsa or acridine orange staining was performed by the method of Piéchaud (23). Preparation of specimens for electron microscopy was made by the method of Ryter and Kellenberger (27). Some samples were embedded in Epon and successively poststained with uranyl acetate and lead citrate.

**Chemical measurements.** Deoxyribonucleic acid (DNA) was measured by the method of Burton (7). The orcinol method used for measuring ribonucleic acid (RNA) was as described by Umbreit et al. (30). Proteins were measured by the Folin method by the method of Sutherland et al. (29). Phospholipids were estimated as phosphate after ashing as described by Ames (2).

**Extraction and separation of phospholipids.** Samples of culture (minimum of 200 ml) at a cell density of about $4.10^8$/ml were chilled, centrifuged at 10,000 × *g*, and washed with cold mineral salts medium 132. The sedimented cells were extracted three times with chloroform-methanol (2:1), by the method of Kanemasa et al. (14). The combined extract was evaporated under nitrogen, redissolved in chloroform-methanol (2:1), and washed three times with 0.3% NaCl. Individual phospholipids were separated by thin-layer chroamtography on thin-layer chromatography plates of Silica Gel 60 $F_{254}$ (E. Merck, A. G., Darmstadt, Germany). One-dimensional separation was performed by the method of Onishi (20) with chloroform-methanol-acetic acid (70:30:10) as the solvent in a saturated atmosphere. The solvents for chromatography were all reagent grade or designated for chromatographic use (e.g., chloroform, obtained from Union Chimique Belge, Brussels, Belgium).

The phospholipids were identified by their chromatographic behavior compared with that of commercial standards: cardiolipin (Koch-Light Laboratories Ltd., Colnbrook, England), phosphatidylglycerol (Supelco Inc., Bellefonte, Pa.), and phosphatidylethanolamine (General Biochemicals, Chagrin Falls, Ohio).

Spots were detected with iodine vapor. After iodine sublimation, they were scraped and removed from the plates by aspiration and placed in 1 ml of scintillation fluid in 0.9 ml of the magnesium nitrate solution used in the chemical determination of phosphate.

**Incorporation of labeled precursors.** Fifty microcuries (2 μmol) of [2-$^3$H]glycerol (New England Nuclear Chemicals GmbH, Dreieichenhain, West Germany) or 70 μCi (5 μmol) of [1-$^{14}$C]acetate was added per 100 ml of culture. At the chosen time, samples of the culture were poured on ice, immediately centrifuged at 10,000 × *g* for 10 min, and washed with cold mineral salts medium.

**Preparation of cell membrane fractions.** Cells from 1 liter of culture at an absorbance (660 nm) of 0.400 were extracted and fractionated by the method of Schnaitman (28) but with the following modifications. The discontinuous sucrose gradient was made by successive layering of 8 ml of 70%, 10 ml of 57%, and 10 ml of 26% sucrose in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer ($10^{-2}$ M, pH 7.4). The extract was homogenized carefully in a mechanical Potter-type homogenizer, and 0.8 ml was layered on the top of the gradient. Generally, centrifugation for 90 min at 21,000 rpm/min in an SW25 rotor (Spinco) gave good separation of inner- and outer-membrane fractions. The less-dense inner-membrane fraction is brownish in color and contains 55 to 66% of the total membrane proteins and 95% of the total succinic dehydrogenase activity, which was measured spectrophotometrically with phenazine methyl sulfate and 2,6-dichlorophenolindophenol (33). The collected fractions were diluted with water, centrifuged, and washed once. Final sediments were suspended in a minimum amount of water (0.15 ml) before phospholipid extraction.

**Intracellular *sn*-glycerol 3-phosphate concentration.** Cell mass, intracellular water, and glycerol-phosphate determinations were carried out with 500 ml of culture at an absorbance (660 nm) of 0.400 by the method of Pizer et al. (24). Enzymatic measurements of glycerol-phosphate were carried out spectrophotometrically by the method of Hohorst (13).

## RESULTS

**Growth and morphological features.** When growing exponentially at 37°C in the rich complex medium (medium 853), the mutated cells tend to form filaments. The culture appears as a mixture of cells of quite different length, some of them being more than 10 times longer than the parental cells. The defect of septation is not total; from time to time, some septa do go to completion. Parent and mutant strains grow exponentially to the same extent, although mutant doubling times are 5 min longer than the corresponding parental ones (e.g., 33 and 28 min for strains KL16-72 and KL16; 32 and 27 min for strains RC10-9 and RC10).

Measurements of absorbance and viable cell counts (Fig. 1a) show that the defect is most pronounced during the exponential phase of growth. The ratio absorbance/number of cells, an estimation of the mean mass per cell, is at least doubled for the mutant. A fivefold increase in this ratio has been observed for cells growing in a turbidostat on the same rich medium maintained for 4 h at an absorbance (660 nm) of 0.250. After a transfer from broth to mineral medium (not shown), filaments divide once or twice at approximately 20-min intervals without any increase in mass, and growth resumes after several hours. Very long cells do not survive the transfer.

When growth occurs at 30°C, instead of at 37°C on the same rich medium (853), the mean length of the mutated cells is diminished but is still slightly higher than the parental one (Fig. 1b). Doubling times at that temperature are 50 and 46 min for strains KL16-72 and KL16, respectively.

When growing at 37°C in a mineral salts-glucose medium, the mutated cells have a nearly normal size. In this medium, the ratio defined above does not differ significantly between the parent and mutant (Fig. 1c), where doubling times are 58 and 77 min, respectively.

Microscopy observations of filamentous cells with ultraviolet fluorescence after nuclear coloration by eosin-Giemsa or acridine orange reveal that the only visible defect is the absence of septation; the nuclear bodies appear to be evenly distributed along the filaments (Fig. 2a). Electron microscopy of sections from these cells (Fig. 2b through f) shows that the fine structure of the nucleoids, the cytoplasm, and the membranes looks normal. However, several invaginations of cytoplasmic membrane are observed: they appear at random, without symmetry or association with expected sites of division (e.g., midcell), and they do not seem to be followed by any constriction of the outer cell wall (Fig. 2c and d). In the rare cases in which a cross section of a septum in the process of formation is observed (Fig. 2e and f), it appears that the cytoplasmic membrane and the double lamellae of the mucopeptide are already completely closed, although outer-membrane material is only beginning to intrude. Under the same conditions of growth and fixation, the parent cell shows constrictive division and no trace of septum (Fig. 2g). These observations are thus in accord with the recent demonstration (5) that the preservation of septa by the standard Ryter-Kellenberger fixation method is an unusual feature. The mutant septa, where they occur, should thus not be as labile as those of the parent wild-type cells (see Discussion).

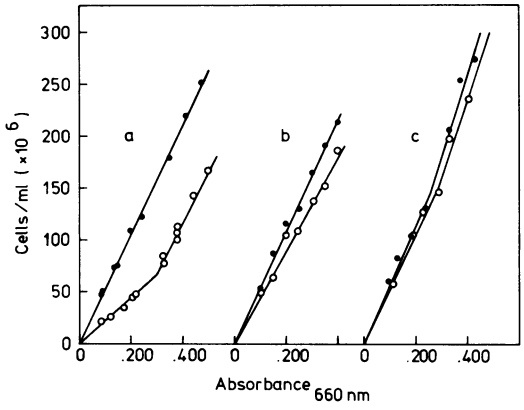


FIG. 1. *Relationship between viable cell counts and absorbance for cultures in complex medium (853) at 37°C (a), 30°C (b), and in mineral salts-glucose medium at 37°C (c). Symbols: ●, KL16 (parent strain); ○, KL16-72 (mutant). Each point is the mean value of four individual counts.*

**Cellular macromolecule contents.** Cellular levels of proteins, RNA, DNA, and phospholipids were measured in a pair of parent and mutant strains (KL16 and KL16-72) growing in the rich medium. The DNA contents were, respectively, 24.0 and 23.9 μg/mg of dry weight with a $\sigma$ value of ±0.3 based on four independent determinations. The RNA/DNA ratio was 10.5 (±0.5), and the protein/DNA ratio was 22 (±1) for the two strains. These values are in agreement with those reported for *E. coli* growing in broth medium (10). The total lipid phosphate was 5.3 (±0.1) μg/mg of dry weight for the two strains. It seems, therefore, that the mutation does not affect the overall synthesis of these fundamental macromolecules; this is reflected in the normal cellular ultrastructure and in the small difference in growth rate between parent and mutant.

**Phospholipid composition.** Although the to-

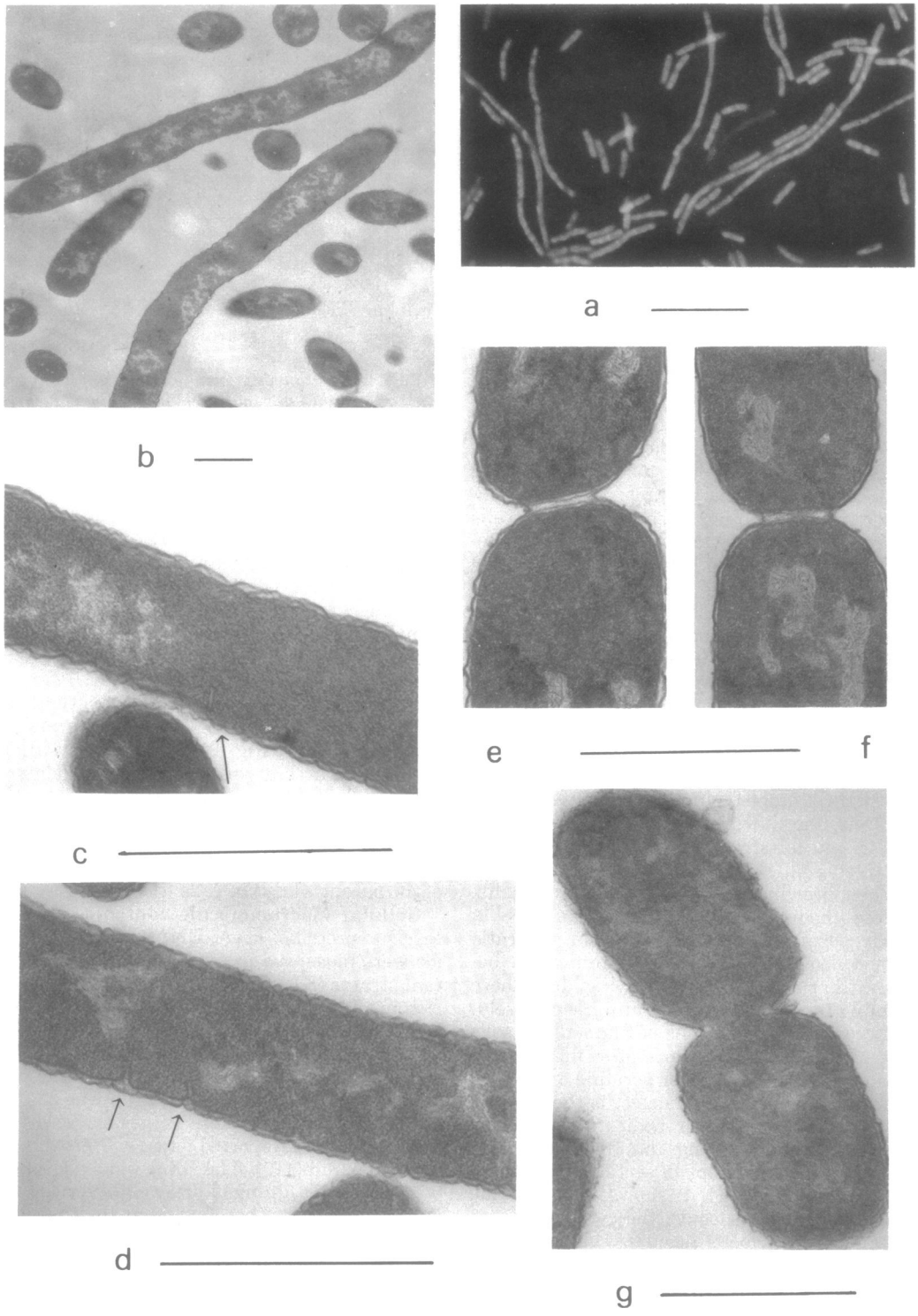


FIG. 2. *E. coli mutant strains. (a) Strain TRF5: ultraviolet fluorescence microscopy, acridine orange coloration; bar represents 10 μm. Sections in electron microscopy, bars represent 1 μm. (b, c, and d) Strain RC10-9. (e and f) Strain KL16-72. (g) Parent strain KL16. All growth was in complex medium 853 at 37°C.*

tal phospholipid content was not altered in the mutant cells growing in filamentous form, preliminary results revealed a clear-cut difference in the distribution of the component cellular phospholipids between a parent and its mutant. Cellular contents of each phospholipid were measured for several independent cultures of four different pairs of strains. The results, expressed as the relative amount of phosphate in each of the three phospholipids, were established with good precision for each strain growing exponentially either in rich medium (Table 2) or in mineral salts-glucose medium (Table 3).

Phosphatidylethanolamine appears in the same high proportion under the two growth conditions for each pair of strains and does not differ significantly from one strain to another. This is in accordance with its known stability under a variety of conditions (14).

For each parent strain examined after growth in complex medium (853), the level of phosphatidylglycerol is at least twice that of cardiolipin; strain KL16 is notably lacking the usual concentration of the latter. In each instance, the presence of the mutation for beta oxidation constitutivity leads to a decrease in the phosphatidylglycerol and an increase in cardiolipin level, so that the ratio of the contents of these two phospholipids drops from the parental level of 2 or more to 1 or less (from 10.0 for strain KL16 to 1.6 for strain KL16-72).

In contrast, after growth in mineral salts-glucose medium where there is no morphological alteration, the ratio of phosphatidylglycerol to cardiolipin is about 1 or less for both parent and mutant. Thus, it appears that the regulatory mutation prevents the cells from attaining the higher level of phosphatidylglycerol, relative to cardiolipin, which is characteristic of the fast growth on rich medium, and has no consequence in glucose-salts medium.

The interpretation of the preceding results as showing an effect of growth rate, rather than simply a nutritional consequence, is supported by the phospholipid distribution obtained after growth on the complex medium at 30°C instead of 37°C. The results are given in Table 2 for strains KL16 and KL16-72. The slowing down of

TABLE 3. *Distribution of phospholipids in pairs of parent and mutant strains after growth in glucose-mineral salts medium at 37°C*[a]

| Strain | Distribution of: | | | Phosphatidylglycerol/cardiolipin ratio |
|---|---|---|---|---|
| | Phosphatidylethanolamine | Phosphatidylglycerol | Cardiolipin | |
| RC10 (parent) | 80 ± 1 | 12 ± 1 | 8 ± 1 | 1.5 |
| RC10-9 (OleR) | 80 ± 1 | 12 ± 1 | 8 ± 1 | 1.5 |
| D5H3G7 (parent) | 75 ± 2 | 13 ± 1 | 12 ± 1 | 1.1 |
| TRF5 (OleR) | 78 ± 2 | 12 ± 1 | 10 ± 1 | 1.2 |
| 37D52 (parent) | 82 | 9 | 9 | 1.0 |
| D52-81 (OleR) | 82 | 7 | 11 | 0.6 |
| KL16 | 77 | 15 | 8 | 1.9 |

[a] Results are expressed as indicated in Table 2, footnote *a*.

TABLE 2. *Distribution of phospholipids in pairs of parent and mutant strains after growth in complex medium (853)*[a]

| Strain | Distribution of: | | | Phosphatidylglycerol/cardiolipin ratio |
|---|---|---|---|---|
| | Phosphatidylethanolamine | Phosphatidylglycerol | Cardiolipin | |
| RC10 (parent) | 74 ± 7 | 18 ± 2 | 8 ± 2 | 2.2 |
| RC10-9 (OleR) | 73 ± 7 | 10 ± 2 | 17 ± 5 | 0.6 |
| D5H3G7 (parent) | 76 ± 3 | 17 ± 1 | 7 ± 2 | 2.4 |
| TRF5 (OleR) | 78 ± 3 | 11 ± 1 | 11 ± 1 | 1.0 |
| 37D52 (parent) | 81 ± 4 | 13 ± 3 | 6 ± 3 | 2.1 |
| D52-81 (OleR) | 80 ± 6 | 9 ± 3 | 11 ± 3 | 0.8 |
| KL16 (parent) | 78 ± 4 | 20 ± 3 | 2 ± 1 | 10.0 |
| KL16-72 (OleR) | 77 ± 4 | 14 ± 2 | 9 ± 2 | 1.6 |
| KL16 (parent) at 30°C | 77 | 20 | 3 | 6.6 |
| KL16-72 (OleR) at 30°C | 76 | 19 | 5 | 3.8 |

[a] Results are expressed as a mean percentage of phosphate in each phospholipid species; the ± number is the $\sigma$ value based on results obtained from a minimum of four individual cultures. All growth was at 37°C unless indicated otherwise.

the growth, which, as we showed above, clearly reduces the cellular division deficiency of the mutants, leads to an intermediate value of the phosphatidylglycerol/cardiolipin ratio in the parent (6.6 compared with 10.0 on complex medium at 37°C and with 1.9 on glucose mineral salts medium at 37°C). It also leads to a much smaller difference of these ratios between parent and mutant strains (6.6 and 3.8). Although the phospholipids are shared almost equally between the inner and the outer membrane of *E. coli* (28), the distribution pattern of the individual phospholipids in these structures needed to be examined. Cells of a pair of strains were extracted and fractionated on a discontinuous sucrose gradient (70%-57%-26%), and the completeness of separation of the outer from the inner membrane was checked by assaying each fraction for succinic dehydrogenase activity. The results (Table 4) show that the high proportion of phosphatidylglycerol relative to cardiolipin, which characterizes the parent strain in fast growth, is found in both membranes and that the decrease of the ratio of these two phospholipids resulting from the mutation affects the two membranes equally.

**Incorporation of [$^3$H]glycerol into cellular phospholipids.** The [$^3$H]glycerol incorporation experiments necessitated the use of strain 8 (8), containing mutations that allow the immediate entry and phosphorylation of glycerol and impede glycerol phosphate oxidation. From this strain, beta oxidation-constitutive mutant 8-01 was derived. First, samples of the culture during growth in the presence of [$^3$H]glycerol were extracted, and the radioactivity and phosphate content of the phospholipid extracts were measured (Fig. 3). The total phospholipid phosphate per mass unit remained fairly constant during culture, as expected for a well-balanced growth, and equivalent values were found for the two strains. In contrast, the specific radioactivity of the phospholipids (radioactivity/phosphate content) is clearly higher and increases faster in the beta oxidation-constitutive strain than in its parent.

This first indication of an increase in turnover in the phospholipids of the mutant was confirmed by similar types of measurements carried out after a 7-min pulse of [$^3$H]glycerol during the exponential phase of growth of the same pair of strains. In this experiment (Table 5), the specific radioactivity of each of the three phospholipids was determined. The reported values were calculated by taking into account the number of glycerol and phosphate moieties in each type of phospholipid. It appears that specific radioactivities of phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin are, respectively, 1.8, 2.3, and 2.3 times higher

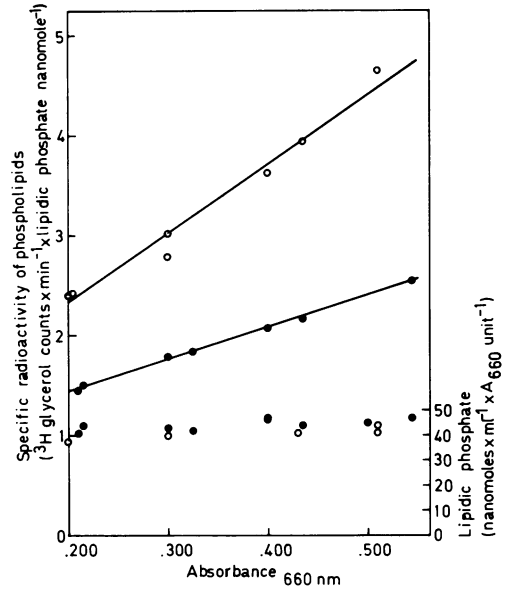


FIG. 3. *Incorporation of [$^3$H]glycerol into total cellular phospholipids during growth, on glucose-Casamino Acids medium, of parent strain 8 (●) and mutant derivative 8-01 (○). Bottom, Total phospholipid phosphate in nanomoles per milliliter per mass unit as a function of growth. Top, Specific radioactivity of total phospholipids during the same growth; [$^3$H]glycerol was added when the culture had attained an absorbance (at 660 nm) of 0.100.*

TABLE 4. *Distribution of phospholipids in inner and outer membranes from a pair of parent and mutant strains*[a]

| Strain | Membrane | Distribution of: | | | Phosphatidylglycerol/cardiolipin ratio |
|---|---|---|---|---|---|
| | | Phosphatidylethanolamine | Phosphatidylglycerol | Cardiolipin | |
| KL16 (parent) | Inner | 73 | 24 | 3.5 | 7 |
| | Outer | 83 | 15 | 1.5 | 10 |
| KL16-72 (OleR) | Inner | 76 | 15 | 8 | 2 |
| | Outer | 81 | 13 | 6 | 2 |

[a] Growth was in complex medium 853 at 37°C; results are expressed as indicated in Table 2, footnote *a*.

for the mutant than the corresponding figures for the parent strain.

**Distribution of pulse [$^3$H]glycerol into membrane phospholipids.** The distribution of the [$^3$H]glycerol incorporated during the 7-min pulse was determined for the three phospholipids of inner and outer membranes; here also, the chemical determinations of the phosphate of each phospholipid permitted calculation of specific radioactivities (Table 6). As expected, the phospholipids of the inner membrane were more highly labeled than those of the outer membrane. The increased incorporation typical of the mutated strain was distributed with the same coefficient for each phospholipid between the two cellular membranes. By taking the specific radioactivity of the phosphatidylethanolamine of the parent strain as unity, it appears that the phosphatidylglycerol from either the inner or the outer membrane of the mutated strain is, by far, the most labeled phospholipid. It may be noted that the cardiolipin of the outer membrane also has relatively high specific radioactivity. This may indicate that the phosphatidylglycerol of the outer membrane (rather than the phoshatidylglycerol of the inner one) could be its immediate precursor.

**Intracellular pool of glycerol phosphate.** The marked increase of specific radioactivities of the phospholipids in the mutant could be due to a lower intracellular level of glycerol phosphate. Although logically this possibility is not compatible with the expected consequences of the constitutivity of the beta oxidation pathway (see Discussion), the cellular content of glycerol phosphate was measured in strains 8 and 8-01 growing exponentially in the same medium that was used in the labeling experiment, but in the absence of glycerol.

Results (Table 7) indicate that the cellular pool is clearly higher (about twice) in the mutant strain, and this is equally true for the other pairs of parent and mutant strains which, unlike the type 8, do not carry any "glycerol" mutation. These additional strains were grown on the rich medium used in the first part of this work. Thus, it seems to be established that the mutation for constitutivity of the beta oxidation

TABLE 5. *Repartition of 7-min pulsed [$^3$H]glycerol into the phospholipids of a pair of parent and mutant strains*[a]

| Strain | Type of phospholipids | Phosphate | | [$^3$H]glycerol (cpm/ml of extract) | Specific radioactivity[b] |
|---|---|---|---|---|---|
| | | nmol/ml of extract | % | | |
| 8 (parent) | Phosphatidylethanolamine | 2,200 | 69 | $553 \times 10^3$ | 251 |
| | Phosphatidylglycerol | 650 | 20 | $343 \times 10^3$ | 264 |
| | Cardiolipin | 340 | 11 | $79 \times 10^3$ | 155 |
| 8-01 (OleR) | Phosphatidylethanolamine | 1,823 | 69 | $823 \times 10^3$ | 451 |
| | Phosphatidylglycerol | 431 | 16 | $524 \times 10^3$ | 608 |
| | Cardiolipin | 385 | 15 | $212 \times 10^3$ | 366 |

[a] Cells were grown on glucose-Casamino Acids medium.
[b] Expressed as counts per minute per glycerol unit and per nanomole of each phospholipid calculated from the phosphate measurements and the known phosphate content of each molecule.

TABLE 6. *Distribution of pulsed [$^3$H]glycerol into the phospholipids of the inner and outer membrane of a pair of parent and mutant strains*[a]

| Strain | Type of phospholipid | Specific radioactivity[b] | | Ratio of specific radioactivities[c] | |
|---|---|---|---|---|---|
| | | Inner membrane | Outer membrane | Inner membrane | Outer membrane |
| 8 (parent) | Phosphatidylethanolamine | 241 | 176 | 1.0 | 1.0 |
| | Phosphatidylglycerol | 311 | 192 | 1.3 | 1.1 |
| | Cardiolipin | 198 | 123 | 0.8 | 0.7 |
| 8-01 (OleR) | Phosphatidylethanolamine | 328 | 266 | 1.4 | 1.5 |
| | Phosphatidylglycerol | 729 | 576 | 3.0 | 3.3 |
| | Cardiolipin | 223 | 326 | 0.9 | 1.8 |

[a] Cells were grown on glucose-Casamino Acids medium.
[b] Expressed as described in Table 5, footnote *b*.
[c] The ratio is based on the specific radioactivity of phosphatidylethanolamine from the inner or the outer membrane of the parent strain taken as unit.

TABLE 7. *Intracellular concentration of sn-glycerol-3-phosphate in four different pairs of parent and mutant strains*[a]

| Strain | Dry wt (mg) | Intracellular water | | Glycerol-P (nmol) | Glycerol-P concn ($\mu$M) | Ratio of glycerol-P concn: (mutant/parent) |
|---|---|---|---|---|---|---|
| | | ml | ml/g of dry wt | | | |
| 8 (parent) | 250 | 0.91 | 3.6 | 396 | 435 | 2.4 |
| 8-01 (OleR) | 270 | 0.84 | 3.1 | 866 | 1,031 | |
| RC10 (parent) | 200 | 0.80 | 4.0 | 320 | 400 | 2.1 |
| RC10-9 (OleR) | 210 | 0.77 | 3.7 | 647 | 840 | |
| 37D52 (parent) | 190 | 0.94 | 4.0 | 500 | 532 | 2.1 |
| D52-81 (OleR) | 213 | 0.68 | 4.0 | 759 | 1,116 | |
| KL16 (parent) | 240 | 1.06 | 4.4 | 472 | 445 | 1.5 |
| KL16-72 (OleR) | 236 | 1.01 | 4.3 | 666 | 659 | |

[a] All values are expressed per liter of culture at an absorbance (660 nm) of 0.400. Strain 8 and strain 8-01 were grown on glucose-Casamino Acids medium; the other pairs were grown on complex medium (853).

alone is responsible for the increased level of intracellular glycerol phosphate.

**Incorporation of [$^{14}$C]acetate into cellular phospholipids.** Strains 8 and 8-01 were included for comparative purposes in [$^{14}$C]acetate labeling experiments, employing procedures similar to those described for [$^{3}$H]glycerol incorporation. Specific radioactivity of the phospholipids from cells grown exponentially in the presence of [$^{14}$C]acetate is constant and equivalent in the two strains; after only one doubling in mass, is a complete steady-state equilibrium in the metabolism of phospholipid acyl moieties reached.

After a pulse of [$^{14}$C]acetate (Table 8) it appears that, although greater labeling of acyl moieties of phosphatidylglycerol is evident, there are no significant differences between parent and mutant strains in the specific radioactivities of their respective phospholipids.

## DISCUSSION

By comparison of results reported recently, it appears that *E. coli* cells grown on rich medium generally contain much more phosphatidylglycerol than cardiolipin, whereas after growth on a mineral salts-glucose medium they contain an equivalent percentage of phosphatidylglycerol and cardiolipin (18). The cellular content of phosphatidylethanolamine, on the other hand, remains the same under various growth conditions.

The systematic study performed here, based on chemical assays of the phospholipids of different K-12 strains grown on two different media, clearly establishes that the ratio of phosphatidylglycerol to cardiolipin is higher in rich medium. That the value of this ratio is related to the growth rate rather than simply being a nutritional consequence is supported by the observation of an intermediate value of the phospholipidic ratio in cells growing in the rich medium at 30°C instead of 37°C. The possibility of regulation of phospholipid metabolism is also suggested by the results obtained here with strains genetically constitutive for the fatty acid beta oxidation pathway. Evidently the mutants tested cannot reach the parental ratio of phosphatidylglycerol/cardiolipin peculiar to the fast-growth conditions, since the postulated regulation of phospholipid metabolism is somehow perturbed in the mutants.

The biosynthetic relationship between the cellular phospholipids of *E. coli* is the following:

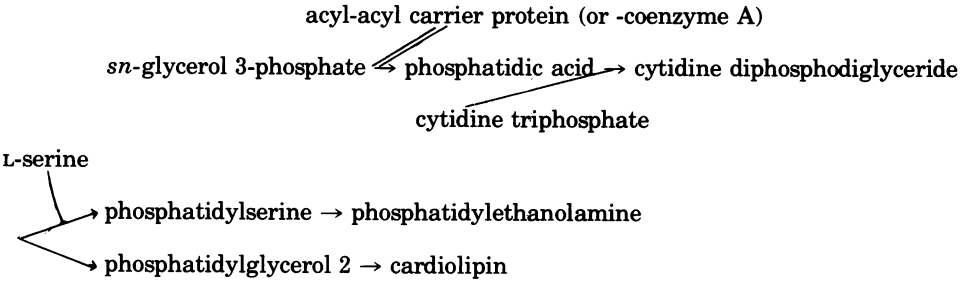

TABLE 8. *Distribution of 7-min pulsed [$^{14}$C]acetate into the phospholipids of strains 8 and 8-01*[a]

| Strain | Type of phospholipids | Specific radioactivity[b] |
|---|---|---|
| 8 (parent) | Phosphatidylethanolamine | 287 |
| | Phosphatidylglycerol | 565 |
| | Cardiolipin | 318 |
| 8-01 (OleR) | Phosphatidylethanolamine | 294 |
| | Phosphatidylglycerol | 580 |
| | Cardiolipin | 440 |

[a] Grown on glucose-Casamino Acids medium.
[b] Expressed as counts per minute per nanomole of phospholipid, calculated as described in Table 5, footnote *b*.

It is well known (for a review, see reference 9) that a decrease of phosphatidylglycerol in favor of cardiolipin occurs as a consequence of numerous manipulations and even normally during transition from the exponential to the stationary growth phase. In fact, all of these conditions have in common a general physiological consequence: the interruption of septation, whether accompanied or not by a slowing down of the growth.

If one can obtain this biochemical variation of the phosphatidylglycerol and cardiolipin levels with the same structural and physiological consequences, namely, the inhibition of septation, but in a quite different way and in energy nonrestrictive conditions, then one could assume that the ratio of these two phospholipids is, in fact, a normal cellular signal of coordination between growth rate and ability to form septa.

It is not likely that the mutants studied in our investigation, growing exponentially in rich medium with a doubling time of about 30 min, suffer from any shortage of energy (in the form of adenosine 5'-triphosphate, e.g., see reference 3). Furthermore, the observation that the phospholipid modification, together with the septation deficiency, disappears in slower growth conditions tends to support the above-mentioned hypothesis. According to Cronan and Vagelos (9), the change of phosphatidylglycerol/cardiolipin ratio is a consequence of a decrease of cellular phosphorylating ability. To explain the results mentioned above, it must be assumed that cardiolipin synthesis, which ocurrs by the condensation of two molecules of phosphatidylglycerol (12), is independent of the cellular level in phosphatidylglycerol and would thus function at phosphatidylglycerol saturation.

[$^{3}$H]glycerol incorporation measurements, coupled with the chemical assays of the various phospholipids, permitted us to determine the specific radioactivity of each molecular species in the appropriate pair of strains. Higher specific radioactivities cannot be due to an increased rate of synthesis. Moreover, phospholipid content per mass unit is constant and equivalent during the growth of parent and mutant strains; thus, the results clearly reveal a higher turnover activity of the glycerol moiety of the phospholipids in the mutants. This suggests that adjustment of phospholipid distribution depends on modulation of such turnover activities, phosphatidylglycerol being the favorite target of these activities. It was shown previously that the rate of turnover of phosphatidylglycerol, especially, varies during the cell life cycle (19).

The preferential turnover of phosphatidylglycerol reinforces the assumption that the rate of production of cardiolipin is largely independent of the phosphatidylglycerol cellular concentration.

The identification of the enzymes responsible for the turnover should lead to an understanding of how the beta oxidation constitutivity can modify their control. Nevertheless, assuming that these turnover activities, when abnormally increased, account for the alteration of the phospholipid pattern and since they seem to be more effective at a higher rate of growth, they ought to be dependent on metabolites or effectors whose cellular concentration changes with growth rate.

The permanent presence of the enzymes of catabolism of the acyl-coenzyme A derivatives conceivably could lower the level of these partners of the glycerol phosphate in the first reaction of phospholipid biosynthesis and thus could lead to some accumulation of glycerol phosphate. A larger cellular pool of glycerol phosphate is actually found in constitutive strains; moreover, this implies that the total internal turnover activity of the phospholipid glycerol moiety is, in fact, still higher in the mutant than shown by radioactivity measurements alone. Therefore, the two-times-higher pool of glycerol phosphate in the mutant implies that the turnover of phosphatidylglycerol, for instance, is in fact about five times higher in the constitutive strain than in its parent.

Incorporation studies with [$^{14}$C]acetate, carried out and expressed in the same way as for [$^{3}$H]glycerol, give quite different results. It appears that the increased turnover of the glycerol portion of the phospholipids does not coincide with an increased turnover in the acyl chains. This would imply that the turnover does not involve free acyl chains but, perhaps, phosphatidic acid. Alternatively, the free acyl chains are involved and could be reutilized as such in the phospholipid metabolism or catabo-

lized without being reequilibrated with the radioactive acetyl coenzyme A precursor in their biosynthesis. Here again, the nature of the enzymatic turnover activities acting on phospholipids should answer the question.

As regards the septation deficiency, so remarkably coincident with the phospholipid alterations, the electron micrographs show that the defect is similar to the one described by Burdett and Murray for two other chain-forming strains of *E. coli* (D22 *envA*; CTR97) (5). In line with the interpretation given by these authors, this would mean that some autolysin activities are defective in our mutants. Mapping results obtained in our laboratory show that the mutation studied here is a point mutation affecting no gene other than the lipid regulation one. Thus, one could hypothesize the necessity of a given ratio of phosphatidylglycerol/cardiolipin to ensure high enough lysin activity for high septum frequency and so correlate this to the phospholipid alteration. The recent work of Goodell et al. (11), showing an enrichment in phosphatidylglycerol of the cell poles in comparison with that of the lateral wall in *E. coli* K-12, gives some support to this assumption. In connection with this, we should like to mention that cells of strain KL16, which contain a high phosphatidylglycerol/cardiolipin ratio, are morphologically thick-set rods, noticeably shorter than other K-12 cells we have observed routinely.

We have seen that the modification of the ratio of phospholipids is similar in the inner and the outer membrane of the mutants, and that, even after a short pulse of glycerol, the specific radioactivity of the phospholipids increases in the same way in both membranes. Since it has been reported that the enzymes involved in phospholipid synthesis are bound to the inner membrane (9), our results show that an exchange of phospholipids occurs rapidly and is unperturbed in the mutant.

The model for septation process proposed by Zaritsky and Pritchard (34) is based on difference in the mode of growth of the various envelope layers. Recently, permeability studies of chain-forming mutants of *envA* type have been interpreted by assuming unbalanced growth of the cellular envelopes, thus supporting this model (17). Initially, an alteration in the growth mode of one or the other of the envelopes does not seem to occur in our mutants. Nevertheless, a proper local phospholipid composition of the outer membrane could be necessary to allow an interaction with the lipid portion of the lipoprotein M, which is known to be covalently linked by its other end to the peptidoglycan (4). This interaction could be crucial during septum formation.

Further studies have to take into account possibilities of such mechanistic, as well as enzymatic, control effects of the membrane phospholipids on the septation process.

## ACKNOWLEDGMENTS

We are grateful to R. G. E. Murray, R. Lavallé, and H. C. Reeves for their helpful suggestions and corrections in the redaction of the manuscript. We thank also J.-P. ten Have for photographic reproduction work.

## LITERATURE CITED

1. **Allen, J. S., C. C. Filip, R. A. Gustafson, R. G. Allen, and J. R. Walker.** 1974. Regulation of bacterial cell division: genetic and phenotypic analysis of temperature-sensitive, multinucleate, filament-forming mutants of *Escherichia coli*. J. Bacteriol. 177:978–986.
2. **Ames, B. A.** 1966. Assay of inorganic phosphate, total phosphate and phosphatases, p. 115–118. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
3. **Bagnara, A. S., and L. R. Finch.** 1973. Relationships between intracellular contents of nucleotides and 5-phosphoribosyl 1-pyrophosphate in *Escherichia coli*. Eur. J. Biochem. 36:422–427.
4. **Braun, V., and K. Rehn.** 1969. Chemical characterization, spatial distribution and functions of a lipoprotein (murein-lipoprotein) of the *Escherichia coli* cell wall. The specific effect of trypsin on the membrane structure. Eur. J. Biochem. 10:426–438.
5. **Burdett, I. D. J., and R. G. E. Murray.** 1974. Septum formation in *Escherichia coli*: characterization of septal structure and the effects of antibiotics on cell division. J. Bacteriol. 119:303–324.
6. **Burdett, I. D. J., and R. G. E. Murray.** 1974. Electron microscope study of septum formation in *Escherichia coli* strains B and B/r during synchronous growth. J. Bacteriol. 119:1039–1056.
7. **Burton, K.** 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315–323.
8. **Cozzarelli, N. R., J.-P. Koch, S. Hayashi, and E. C. C. Lin.** 1965. Growth stasis by accumulated L-α-glycerolphosphate in *Escherichia coli*. J. Bacteriol. 90:1325–1329.
9. **Cronan, J. E., Jr., and R. P. Vagelos.** 1972. Metabolism and function of the membrane phospholipids of *Escherichia coli*. Biochim. Biophys. Acta 165:25–60.
10. **Forchhammer, J., and L. Lindahl.** 1971. Growth rate of polypeptide chains as a function of the cell growth rate in a mutant of *Escherichia coli* 15. J. Mol. Biol. 55:563–568.
11. **Goodell, E. W., U. Schwarz, and R. M. Teather.** 1974. Cell envelope composition of *Escherichia coli* K12: a comparison of the cell poles and the lateral wall. Eur. J. Biochem. 47:567–572.
12. **Hirschberg, C. B., and E. P. Kennedy.** 1972. Mechanism of the enzymatic synthesis of cardiolipin in *Escherichia coli* (phosphatidylglycerol/glycerol/CDP-diglyceride/isotope distribution). Proc. Natl. Acad. Sci. U.S.A. 69:648–651.
13. **Hohorst, H. J.** 1963. L-(−)-glycerol-1-phosphate determination with glycerol-1-phosphate dehydrogenase, p. 215–219. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis. Academic Press Inc., New York.

14. **Kanemasa, Y., Y. Akamatsu, and S. Nojima.** 1967. Composition and turnover of the phospholipids in *Escherichia coli*. Biochem. Biophys. Acta 144:382-390.
15. **Klein, K., R. Steinberg, B. Fiethen, and P. Overath.** 1971. Fatty acid degradation in *Escherichia coli*. An inducible system for the uptake of fatty acids and further characterization of *old* mutants. Eur. J. Biochem. 19:442-450.
16. **Normark, S.** 1970. Genetics of a chain-forming mutant of *Escherichia coli*. Transduction and dominance of the envA gene mediating increased penetration to some antibacterial agents. Genet. Res. 16:63-78.
17. **Normark, S., and H. Wolf-Watz.** 1974. Cell division and permeability of unbalanced envelope mutants of *Escherichia coli* K 12. Ann. Microbiol. (Paris) 125:211-226.
18. **Nunn, W. D., and B. E. Tropp.** 1972. Effects of phenethyl alcohol on phospholipid metabolism in *Escherichia coli*. J. Bacteriol. 109:162-168.
19. **Ohki, M.** 1972. Correlation between metabolism of phosphatidylglycerol and membrane synthesis in *Escherichia coli*. J. Mol. Biol. 68:249-264.
20. **Onishi, Y.** 1971. Phospholipids of virus-induced membranes in cytoplasm of *Escherichia coli*. J. Bacteriol. 107:918-925.
21. **Overath, P., G. Pauli, and H. U. Shairer.** 1969. Fatty acid degradation in *Escherichia coli*. An inducible acyl-CoA synthetase, the mapping of *old*-mutations, and the isolation of regulatory mutants. Eur. J. Biochem. 7:559-574.
22. **Pages, J. M., M. Piovant, A. Lazdunski, and C. Lazdunski.** 1975. On the control of septation in *Escherichia coli*. Biochimie 57:303-313.
23. **Piéchaud, M.** 1954. La coloration sans hydrolyse du noyau des bactéries. Ann. Inst. Pasteur Paris 86:787-793.
24. **Pizer, L. E., J. P. Merlie, and M. Ponce de Leon.** 1974. Metabolic consequences of limited phospholipid synthesis in *Escherichia coli*. J. Biol. Chem. 249:3212-3224.
25. **Ricard, M. and Y. Hirota.** 1973. Process of cellular division in *Escherichia coli*: physiological study on thermosensitive mutants defective in cell division. J. Bacteriol. 116:314-322.
26. **Rodolakis, A., F. Casse, and J. Starka.** 1973. Morphological mutants of *Escherichia coli* K 12. Mapping of the *envC* mutation. Mol. Gen. Genet. 130:177-181.
27. **Ryter, A., and E. Kellenberger.** 1958. Etude au microscope électronique de plasmas contenant de l'acide desoxyribonucléique. I. Les nucléoides des bactéries en croissance active. Z. Naturforsch. 13B:597-605.
28. **Schnaitman, C. A.** 1970. Protein composition of the cell wall and cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. 104:890-901.
29. **Sutherland, E. W., C. F. Cori, R. Haynes, and N. S. Olsen.** 1949. Purification of the hyperglycemic-glycogenolytic factor from insulin and from gastric mucosa. J. Biol. Chem. 180:825-837.
30. **Umbreit, W. W., R. H. Burris, and J. F. Stauffer.** 1959. Manometric techniques, p. 274. Burgess Publishing Co., Minneapolis.
31. **Vanderwinkel, E., and M. De Vlieghere.** 1968. Physiologie et génétique de l'isocitrate et des malate synthases chez *Escherichia coli*. Eur. J. Biochem. 5:81-90.
32. **Vanderwinkel, E., M. De Vlieghere, and J. Vanden Meersche.** 1971. Mutations habilitant *Escherichia coli* á croître sur acides gras moyens. Eur. J. Biochem. 22:115-120.
33. **Weeger, C., D. V. Der Vartanian, and W. P. Zeylemaker.** 1969. Succinate dehydrogenase, p. 81-90. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 13. Academic Press Inc., New York.
34. **Zaritsky, A., and R. H. Pritchard.** 1973. Changes in cell size and shape associated with changes in the replication time of the chromosome of *Escherichia coli*. J. Bacteriol. 114:824-837.