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Cytoplasmic Membrane Fraction That Promotes Septation in an *Escherichia coli lon* Mutant

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A particulate fraction derived from bacterial cells stimulates septation in irradiated *Escherichia coli lon* mutants when added to postirradiation plating media. It was established that the particles are derived from the cytoplasmic membrane and that they have been partially purified by sucrose density gradient centrifugation. These particles also contain the cytochrome-based respiratory activity of the cell. A variety of experiments established a correlation between the septation-promoting activity of the particles and their ability to remove oxygen from the postirradiation plating medium. It was suggested that the efficient removal of oxygen from the medium allowed the *lon* cells to repair radiation-induced damage to the septation mechanism.

In 1956, DeLaporte made the observation that the survival of UV-irradiated *Escherichia coli* B cells, judged by the development of microcolonies on a microscope slide, was improved in regions where the cells were close to each other (6). In less dense regions, the irradiated cells grew but did not form septa. As a result, long multinucleate filaments were formed that did not develop into colonies. DeLaporte suggested that some material might be moving from donor cells to recipient cells and improving survival. She called the phenomenon "neighbor restoration." In earlier studies, we developed a quantitative assay for neighbor restoration and demonstrated that the phenomenon is only observed in recipient cells that have a mutation at the *lon* locus (1-3). We and others have also established that septation and subsequent recovery of colony-forming ability in *lon* mutants can be stimulated by a variety of agents (3, 5). The presence of live donor cells is not required.

One of the most effective agents for promoting septation and recovery in *lon* mutants is a particulate fraction that can be obtained from some microorganisms and other cells. Various properties of this particulate material have been previously described (7). In this report, we provide evidence that the particles are derived from the cytoplasmic membrane of the donor cells and that the septation-promoting activity is related to the ubiquinone-cytochrome-dependent respiratory activity also associated with these preparations.

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MATERIALS AND METHODS

Organisms and growth conditions. *E. coli* AB1899nm was used as the recipient test organism. It is a streptomycin-resistant, nonmucoid derivative of the *lon* mutant isolated by Howard-Flanders et al. from *E. coli* AB1157 (10). If cells from stationary-phase AB1899nm cultures are exposed to ionizing radiation, they form very long multinucleate nonseptate filaments. The usual source of septation-promoting activity was a streptomycin-sensitive *E. coli* B/r ORNL isolate previously described by Adler and Haskins (4). The *E. coli* K-12 ubiquinone and menaquinone mutants were supplied by I. G. Young. Strains AN385 (*ubi men*$^+$), AN386 (*ubi*$^+$ *men*), and AN387 (*ubi*$^+$ *men*$^+$) are isogenic transductants and streptomycin resistant. The phenotypes of the mutants were confirmed by analyzing their growth requirements, the enzymatic characteristics of their membranes, and their ubiquinone content (17). Other organisms used as sources of septation-promoting activity are described in the appropriate parts of the text.

The liquid medium used for most experiments was Difco nutrient broth supplemented with yeast extract (0.5%) and sodium chloride (0.6%). Glucose, when used, was present at 0.4%. Organisms were diluted 1/1,000-fold and grown at 37°C with shaking. The quinone mutants were grown in double-strength medium 56 with glucose at 0.5% and Casamino Acids at 0.1% (13). The plating medium used was Difco nutrient agar supplemented with yeast extract (0.5%), glucose (0.04%), and streptomycin (60 μg/ml). Anaerobic incubation was achieved by placing petri dishes in Torbal containers (model AJ-2, Torsion Balance Co.) and alternately pulling a vacuum and flushing with high-purity nitrogen for 10 cycles. The containers were then incubated overnight at 37°C.

Irradiation of test organism and assay procedure. A 1:1,000 dilution (in 0.85% NaCl) of a stationary-phase culture of AB1899nm grown in nutrient

medium supplemented with 0.4% glucose was exposed to approximately 25 krads (Cobalt 60) in a Gamma cell 200 (Atomic Energy of Canada, Ltd.). This dose inhibits septum formation in approximately 99% of the cells. Immediately after irradiation, the cells were diluted into supplemented nutrient agar held at 42 to 45°C. Ten-milliliter samples of molten agar containing irradiated cells were pipetted into petri dishes (60 by 15 mm) to which material being tested for septation-promoting activity had been added. (The volume of material added did not exceed 0.4 ml.) Control plates were made without test material. The contents of the plates were mixed and, as soon as the agar had hardened, incubated overnight at 37°C either aerobically or anaerobically. Colonies were counted after 18 to 24 h.

In experiments in which only small amounts of material were available for testing, the assay was carried out in 1-ml quantities of agar placed in the 2-ml wells of a multiwell Disposo-Tray (Flow Laboratories, Inc., no. 76-300-05).

Preparation of septation-promoting factor. The procedure used was similar to that used by other investigators for the preparation of membrane fractions (15). *E. coli* B/r ORNL cells were grown to stationary phase in nutrient broth supplemented with yeast extract and glucose or to late log phase without glucose supplementation of the medium. Septation-promoting factor is present in cells grown under both conditions, but the separation of cytoplasmic membrane from outer membrane, as described below, is more efficient when performed on the log-phase cells. Cells were harvested and washed with cold HEPES buffer, pH 7.5 (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). Cell pellets were frozen until needed. Ten grams of cell pellet was resuspended with 25 ml of HEPES buffer and subjected to 1 min of stirring with a Sorvall Omnimixer operated at full speed. The suspension was centrifuged for 15 min at 3,000 × *g* at 0°C, and the supernatant was discarded. The pellet was resuspended in 25 ml of HEPES buffer, and 1 mg each of DNase I and RNase A (Sigma Chemical Co., St. Louis, Mo.) were added. The suspension was passed through a French press two or three times at 20,000 lb/in^2. It was incubated in an ice bath for 1 h, and then $MgCl_2$ was added to achieve a final concentration of 0.16 mM. After an additional 1-h incubation, it was centrifuged for 15 min at 12,000 × *g* at 0°C. The supernatant from this centrifugation is referred to as "crude extract" in the text and figure legends. For many experiments, this supernatant was centrifuged for 5 h at 175,000 × *g* at 5°C. The pellet from this high-speed centrifugation was resuspended in 25 ml of HEPES buffer (approximately 20 to 30 mg [dry weight] per ml). It is referred to as "crude membrane fraction" and contains the septation-promoting activity. It corresponds closely to the "P_2" fraction described in our earlier work (7).

Separation of membrane fractions. Cytoplasmic membrane was separated from outer membrane and the peptidoglycan layer by sucrose gradient procedures described by Schnaitman (15). Both continuous and discontinuous gradients were used, but most data were collected from discontinuous gradients. One-milliliter quantities of crude membrane fraction were layered onto 38-ml gradients and centrifuged for 16 h at 95,000 × *g* in a swinging- bucket rotor at 5°C. Gradients were harvested in approximately 1-ml fractions by use of a Buchler Auto Densi-Flow and polystatic pump and an Isco model 1200 fraction collector. Fractions to be assayed for enzymatic and septation-promoting activity were used either immediately or after frozen storage.

Enzyme activity assays and protein determinations. Assays for NADH oxidase and succinic dehydrogenase were done by procedures described by Osborn et al. (14). Cytochrome difference spectra were performed by the method of Schnaitman (15). Protein was determined on trichloroacetic acid precipitates by the Lowry method (12).

Determination of oxygen removal rates. The YSI model 53 biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) was used to measure the rate at which whole cells, cell extracts, and crude membrane preparations could remove oxygen from nutrient broth and other solutions. The monitor was operated at 37°C. In most experiments, the substrate was 3 ml of liquid that had initially been equilibrated with air at 37°C. Oxygen removal was recorded as the percentage of oxygen removed per minute.

Inactivation of respiratory activity and septation-promoting activity by 331- and 334-nm radiations. Crude membrane fractions were exposed in quartz cuvettes to radiation from a Hilger quartz prism monochromator (Hilger and Watts, Inc., Morton Grove, Ill.) illuminated by a 1,000-W Phillips high-pressure mercury arc lamp (North American Phillips Corp., Hightstown, N.J.). Radiation was passed through a thin Mylar film to eliminate scattered, short wavelengths. The average exposure rate at the center of the sample was 50 J/m^2 per s for a 313-nm radiation and 46 J/m^2 per s for a 334-nm radiation. Light of these wavelengths has previously been shown to interfere with the activity of the cytochrome system (11, 18). Immediately after exposure, samples were assayed for their ability to remove oxygen from nutrient broth and to promote septation.

RESULTS

Promotion of septation by cell extracts. Figure 1 illustrates the results of a typical assay for septation-promoting activity. The *E. coli* AB1899 cells were exposed to a dose of 25 krads. After appropriate dilution, plating, and incubation, approximately 25 colonies per plate developed in the absence of crude membrane fraction. This represents a survival of 0.5% when compared with an unirradiated control. In the presence of optimum amounts of membrane fraction, more than 800 colonies per plate developed. This represents an increase in survival to approximately 15%. Microscopic observation of irradiated cells growing on the plating medium during the first few hours after irradiation reveals that in the absence of membrane fraction most cells grow into long, nonseptate, multinucleate fila-

ments that do not give rise to colonies. In the presence of the fraction, the irradiated cells begin to grow into filaments but after several hours initiate septation and finally give rise to macroscopic colonies (2, 3). The assay was much more sensitive if the incubation of the nutrient agar containing test cells and crude membrane fraction was carried out under relatively anaerobic conditions (Fig. 1). This is not due simply to an oxygen sensitivity of the active material. Experiments not shown here establish that O_2 can be passed through crude or partially purified membrane fractions for several hours at 37°C without affecting subsequent septation-promoting activity. One possible explanation for the enhancement of the assay by anaerobic incubation is presented below.

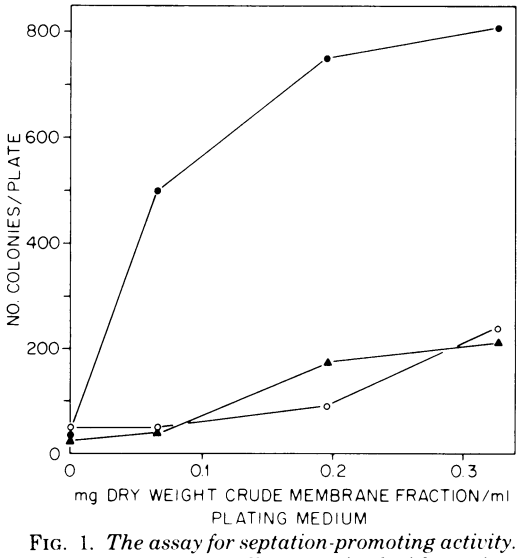

FIG. 1. *The assay for septation-promoting activity. Irradiated AB1899nm cells were mixed with nutrient agar containing the indicated amounts of filter-sterilized crude membrane fraction derived from E. coli B/r cells. Symbols: ●, plates incubated anaerobically; ○, plates incubated aerobically; ▲, plates incubated in a mixture of nitrogen and 10% oxygen.*

Septation-promoting activity is not the direct product of the *lon*$^+$ gene. Both the *lon* mutant and its wild-type parent contain the active material at somewhat lower concentrations than *E. coli* B/r. We have also found septation-promoting activity in the filaments formed by *lon* cells after exposure to radiation. The activity found in *lon* cells and filaments seems to be less stable than that found in *lon*$^+$ cells, but our information on this point is less than satisfactory. Some preparations have lost their activity rapidly, whereas others have not.

Association of septation-promoting activity and the cytoplasmic membrane. Our earlier experiments had established that septation-promoting activity was associated with a particulate fraction that was insensitive to RNase, DNase, and several proteolytic enzymes, but sensitive to lipase, phospholipase A, and detergents (7). This suggested that the activity might be associated with the cell envelope. For this reason, we began to prepare and separate cell membrane fractions by well-established methods (15). Figure 2 presents data from a sucrose density gradient experiment demonstrating that essentially all of the septation-promoting activity was found in association with the cytoplasmic membrane.

In a gradient of this composition, the material accumulating at a density of 1.161 is known to consist primarily of cytoplasmic membrane; the material at 1.224 is primarily outer membrane and the peptidoglycan layer (15). In Table 1, we confirmed that some enzymes known to be relatively concentrated in the cytoplasmic membrane were indeed found in higher concentration

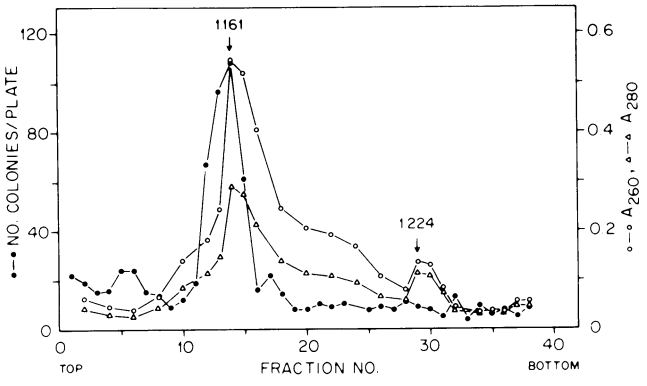

FIG. 2. *Distribution of cell membrane fractions and septation-promoting activity in a discontinuous sucrose gradient (see text). The arrows indicate the location and density (20°C/4°C) of the two visible bands. The upper band is narrow and red-brown. The lower band is more diffuse and opaque white.*

TABLE 1. *Enzymatic activities of fractions separated on a discontinuous sucrose gradient*

Material	Density (20°C/4°C)	Protein (mg/ml)	NADH oxidase[a]	Succinic dehydrogenase[a]
Crude membrane function		14.5	2.06	1.16
Cytoplasmic membrane	1.161	1.0	20.97	5.28
Outer membrane	1.224	0.8	3.63	1.71

[a] Enzyme activities are reported as micromoles per minute per milligram of protein.

in the fraction with a peak density at 1.161. We have also observed that there is a strong absorption peak at approximately 550 nm when a difference spectrum is performed on the presumed cytoplasmic membrane material. No such peak is found for the outer membrane material. This corresponds to the distribution of cytochromes previously reported (15).

If the fractions from the cytoplasmic membrane region of a sucrose density gradient are pooled, concentrated, and centrifuged in a gradient of slightly different composition, it is possible to separate much of the material that absorbs at 260 and 280 nm from the septation-promoting activity. The material remaining at a density of 1.161 retains most of the septation-promoting activity, is dark red-brown, and absorbs light strongly at 412 nm.

Numerous unsuccessful attempts to solubilize the septation-promoting activity have been made. In our efforts to achieve further purification, a variety of detergents, organic solvents, enzymes, and mechanical techniques in various combinations have either destroyed the activity or left it bound to the membrane fraction. For this reason, we have postponed attempts at further purification of the septation-promoting activity and have instead concentrated on establishing some related properties of the cytoplasmic membrane.

Septation-promoting activity and the cytochrome system. Because we were impressed with the coconcentration of septation-promoting activity and the dark red-brown material in sucrose density gradient fractions, we considered the possibility that the cytochrome system might play a direct role in promoting septation. The most obvious and easily measured activity of the cytochrome system is the reduction of oxygen to water. We, therefore, made measurements of the rate at which septation-promoting fractions removed oxygen from nutrient broth having the same constituents as the plating medium used in the septation assay, except for the omission of agar.

We observed that a typical crude membrane fraction, such as that used to produce the data for Fig. 1, removed oxygen from nutrient broth at a rate of 18%/min when present at a concentration of approximately 1 mg/ml. If no oxygen was added to the broth during the reaction period, essentially all of the initially dissolved oxygen was removed in 5 min. This reaction was sensitive to 10 mM sodium cyanide.

A variety of membrane fractions that lack the septation-promoting activity also lack the ability to remove oxygen from nutrient broth. The data presented in Fig. 3 show that a membrane preparation made from a culture of *E. coli* B/r cells grown under anaerobic conditions lacks the septation-promoting activity. It also is incapable of removing oxygen from nutrient broth. Extracts were also prepared from *Bacillus subtilis*, *Streptococcus faecalis*, *Rhodospirillum rubrum*, and *Pseudomonas aeruginosa*. Only the extract from *P. aeruginosa* had septation-promoting activity. This was the only extract that could remove oxygen from nutrient broth at a significant rate. At a concentration of 1.0 mg (dry weight) of extract per ml of nutrient broth, the oxygen removal rate was 17%/min. The other extracts, at comparable concentrations, removed oxygen at a rate of less than 1%/min.

It has been reported that exposure to light between 300 and 400 nm interferes with the functioning of the cytochrome-based electron transport system (11, 18). This phenomenon is

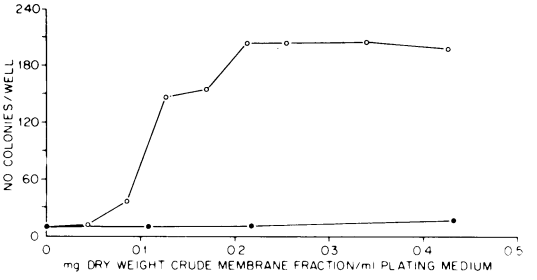

FIG. 3. *Crude membrane fractions from anaerobically grown E. coli B/r cells lack septation-promoting activity and the ability to remove oxygen from nutrient broth. Cultures were grown in a glucose-supplemented (0.4%) nutrient broth in a long glass cylinder fitted with a fine-porosity sintered glass filter in its base. Sterile air of high-purity nitrogen was passed into the culture through this filter during the entire growth period (approximately 20 h). Crude membrane fractions were prepared from both cultures as described in the text. Symbols: ○, membrane fraction from aerobically grown cells; ●, membrane fraction from anaerobically grown cells. At 1.67 mg of crude membrane fraction per ml of nutrient broth, the rate of oxygen uptake for the fraction from anaerobically grown cells was 19%/min and 0.0%/min for the fraction from anaerobically grown cells.*

probably due to the destruction of ubiquinone, an essential part of the system. It has been established that, in the near-UV region, 313 nm is a very effective wavelength for inactivating ubiquinone, and 334 nm is slightly less effective (18). Radiation of 313 nm inactivated the oxygen-consuming and septation-promoting abilities of crude membrane fractions (Fig. 4). The kinetics of inactivation appear to be similar for both processes. Radiation of 334 nm produced similar results but was slightly less effective.

Further evidence for the possible identity of the cytochrome system and the septation-promoting factor came from experiments in which ubiquinone and menaquinone mutants of *E. coli* were used (17). These mutants have undergone genetic alterations at specific loci and, except for quinone content, appear to have membranes that are identical to those from wild-type *E. coli*. An extract from AN385 (*ubi men*$^+$) lacked the ability to remove oxygen from nutrient broth and also did not promote septation (Fig. 5). An extract from AN386 (*ubi*$^+$ *men*) removed oxygen and promoted septation. Likewise, an extract from AN387 (*ubi*$^+$ *men*$^+$) was capable of oxygen removal and septation promotion.

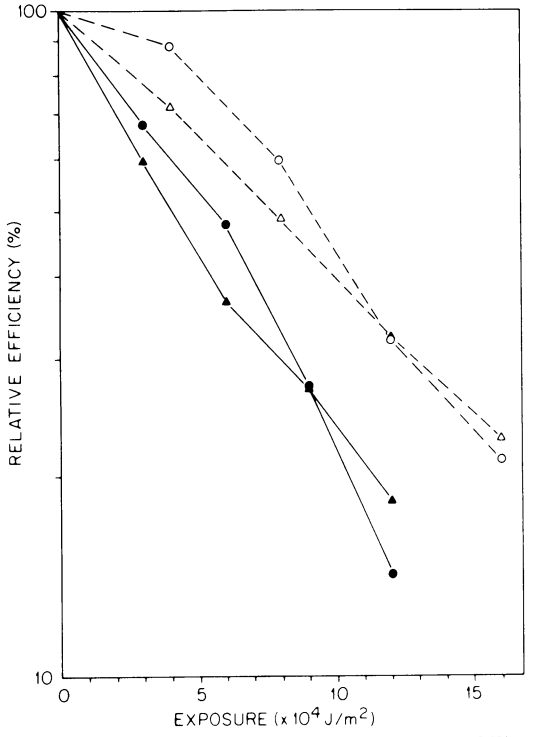

FIG. 4. *Inactivation of oxygen-reducing ability and septation-promoting ability by 313 and 334 nm of radiation. Relative efficiency for oxygen reduction = initial slope of the rate curve for oxygen reduction by the irradiated crude membrane sample/initial slope of the rate curve of an unirradiated sample. Relative efficiency for septation promotion = the concentration of crude membrane fraction required to produce a one-half maximum effect/concentration of the irradiated sample to produce the same effect. Symbols: dotted lines, 334-nm radiation; ○, relative efficiency of oxygen reduction; △, relative efficiency of septation promotion; solid lines, 313-nm radiation; ●, relative efficiency of oxygen reduction; ▲, relative efficiency of sepation promotion.*

DISCUSSION

The experimental results presented are most readily interpreted in the following framework. The septation-promoting activity originally described as a property of intact cells (6) and later shown to be associated with a subcellular particle (7) is now recognized as a part of the cytoplasmic membrane. The ability of the cytoplasmic membrane to promote septation may be a direct function of its ability to remove oxygen rapidly and completely from the medium in which the test cells are embedded.

The membrane-associated, septation-promoting activity is not a direct product of the *lon* locus. The product has recently been shown to be a polypeptide with DNA-binding properties (19). We have some evidence indicating that the septation-promoting factor isolated from *lon* mutants may be less stable than that from *lon*$^+$, but the data are not completely convincing. It is possible that the polypeptide produced by *lon* cells does influence the properties of the cytoplasmic membrane in some subtle way.

The experiments presented in Fig. 3, 4, and 5

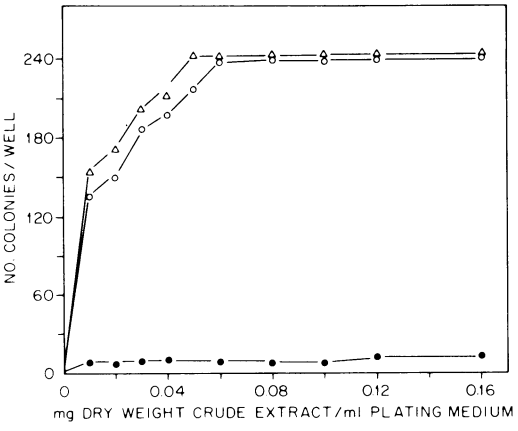

FIG. 5. *Septation-promoting activity and respiratory activity of cell extracts from ubiquinone and menaquinone mutants. Symbols: △, AN387 (ubi*$^+$ *men*$^+$*); ○, AN386 (ubi*$^+$ *men); ●, AN385 (ubi men*$^+$*). The rate of oxygen removal at 0.67 mg (dry weight) of cell extract per ml of nutrient broth was 19%/min for AN387, 18%/min for AN386, and 0.2%/min for AN385.*

provide a variety of evidence supporting the suggestion that rapid and relatively complete removal of oxygen from the plating medium is required for the promotion of septation in irradiated *lon* cells. We do not know what substrate in the complex nutrient medium is being oxidized, but the data shown in Fig. 4 and 5 make it likely that oxidation is taking place via the ubiquinone-cytochrome system rather than the menaquinone pathway.

We have not been able to promote septation by treatment of the plating medium with a variety of N_2 flushing and evacuation regimes or the addition of reducing agents such as sodium thioglycolate or sodium dithionite. Apparently, these treatments do not bring about the rapid and relatively permanent anaerobiosis achieved by the membrane fraction. Measurements made with the Biological Oxygen Monitor demonstrate that N_2 flushing is very ineffective in achieving rapid anaerobiosis of a solid medium. However, removal of the oxygen above the medium does allow low concentrations of the cytoplasmic membrane fraction to be effective in promoting septation (Fig. 1). An earlier report by Hodgkins and Alper (9) demonstrated that, at least under their experimental conditions, postirradiation anaerobiosis achieved by hydrogen flushing of a liquid medium did favor recovery (septation) in irradiated *E. coli* B cells but not *E. coli* B/r cells. Although not known at the time, it is now clear that *E. coli* B is a *lon* mutant and shares many characteristics with the *lon* K-12 strain with which we performed our experiments (8). Hodgkins and Alper observed that the lag phase and subsequent generation time for *E. coli* B cells was increased by anaerobic incubation. They suggested that anaerobiosis, along with several other treatments, promoted recovery by increasing the time available for repair of radiation damage. Microscopic observations of *E. coli* AB1899 cells (*lon*) grown in the presence of membrane fractions do not suggest that any increase in the lag phase or generation time takes place (7; unpublished data). Although possibly related, it is not clear how the current observations should be compared with those of Hodgkins and Alper.

The phenomenon of neighbor restoration originally described by DeLaporte may now be explained in the light of our current results. It appears likely that high densities of cells may have produced, as a result of their metabolic activity, local areas of anaerobiosis in the medium present on the microscope slides. According to our current interpretation, this would have favored the resumption of septation in the irradiated *lon* cells.

We do not know the nature of the radiation-induced lesions in *lon* cells that leads to failure of septation. Likewise, we do not understand how the anaerobiosis achieved by the addition of cytoplasmic membrane fractions to the plating medium stimulates the repair of this damage. As far as is known, the DNA repair pathways that are present in wild-type *E. coli* are also present and functional in *lon* mutants (10, 16).

We currently favor the idea that radiation interferes with septation in *lon* cells by causing a lesion which affects DNA-membrane interaction. We introduce the notion of membrane involvement for two reasons. First, DNA synthesis and repair, per se, seem to proceed normally in irradiated *lon* cells (10, 16). Second, it is attractive to consider that the interaction of DNA and the cytoplasmic membrane is responsible for both the positioning and timing of septation. Postirradiation anaerobiosis may provide the appropriate conditions for the operation of a repair system directed at this particular lesion. Future experiments will be designed to test these ideas.

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