Stable L-Phase of Erwinia carotovora Induced by Ultraviolet Irradiation

E. Cabezas de Herrera, M. Rubio Huertos, and O. García Jurado

Institute of Microbiology "Jaime Ferrán", Consejo Superior de Investigaciones Científicas, Madrid, Spain.
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ABSTRACT

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After having obtained the lethality curve of *Erwinia carotovora* strain 2012 and established the sensitivity of the bacteria to ultraviolet radiation, we found that the minimal sublethal dose of 15 seconds (105 erg/mm^2/sec) was the most suitable. We obtained typical L-phase colonies on agar-serum plates by irradiating the bacteria with that dose when the DNA was in the open-strand state. The colonies were formed by spherical elements with two well differentiated layers: an outer degraded layer and the inner or cytoplasmic membrane. These spheroplasts are less pathogenic than the parental bacteria on *Phaseolus vulgaris* and *Vicia faba*. These forms have been transferred weekly during five years without reversal to the normal bacillary form and they still show the same characteristics. Ten per cent horse serum is necessary for the development of the L-forms, but they are not induced by it.

RESUMEN

Obtenemos la curva de letalidad de la bacteria *Erwinia carotovora* estirpe 2012 con el fin de conocer su sensibilidad a la radiación ultravioleta. La dosis minima subletal que se consigue a los 15 segundos de exposición a una radiación de 105 erg/mm^{2}sec, es la mas apropiada para nuestras experiencias. Irradiando la bacteria con esta dosis, cuando el ADN bacteriano se está dividiendo, hemos obtenido formas L que muestran dos envolturas celu lares bien diferenciadas, una exterior degradada, otra interior o membrana citoplásmica. Estas formas son menos patógenas sobre *Vicia faba* y *Phaseolus vulgaris* que la bacteria de que proceden. Estas formas L han sido resem bradas semanalmente durante cinco años, conservando las mismas características bioquímicas y morfológicas. El suero de caballo añadido al medio, parece ser necesario para el desarrollo de estas formas L, sin embargo no son inducidas por él.

Since the discovery of L-forms in *Streptobacillus moniliformis* by Klienerberger-Nobel (6) an extensive bibliography has developed concerning the induction of bacterial L-forms by the use of penicillin, amino acids and related substances, or high concentrations of electrolyte (7).

Medill and Hutchinson (8) stated that pre-exposure of the bacteria to ultraviolet (UV) light decreased the ability of *Proteus mirabilis* rods to form L-type colonies on penicillin agar, but Rubio Huertos and Cabezas de Herrera (11) obtained permanent L-forms of *Agrobacterium tumefaciens* by UV irradiation and they emphasized the importance of the ability of the bacterial strain to produce the L-form.

Doses of UV light, considerably lower than those required to stop cellular growth and metabolism of nucleic acids, inhibited cell division. Pontefract and Thatcher (9) and Deering (2) considered it more a delay in division than a permanet inhibition.

Hijmans et al. (4) reviewed the literature on the bacterial species which grow in the L-phase. The only reference to L-forms being obtained with UV light was the report by Rubio Huertos and Cabezas de Herrera (1, 11) with *A. tumefaciens*. As far as we know, no L-phase colonies have been obtained by irradiating *E. carotovora* with UV light, although L-phase of *E. carotovora* obtained by penicillin treatment was described by Jones and Paton (5) who also described the association of the *E. carotovora* L-phase with the plant tissues and indicated that the L-phase was reversible to the normal form.

In the current paper, we present the results obtained when we irradiated *Erwinia carotovora* with UV light, wavelengths 2535 Å, using the same conditions that we reported previously (11) for *A. tumefaciens*. The study of L-phase formation in phytopathogenic bacteria is of interest because many diseases are now found to be induced by mycoplasmalike organisms in plants and there is a possible relationship between L-forms and mycoplasmas (10).

MATERIALS AND METHODS

Erwinia carotovora 312 from the "National Collection of Phytopathogenic Bacteria" at Harpenden, England, was used throughout these studies.

For irradiation studies, cells from 18-hour-old synchronous cultures, which were obtained by the method of Scott et al. (16), were sedimented and washed with phosphate buffer (pH 7.0), resuspended in the buffer, adjusted to a concentration of 10^9 cells per ml, and 2 ml of the suspension were placed in a 4.5-cm diameter petri dish. The suspension was exposed to UV radiation.

Irradiation.—Ultraviolet radiation was delivered by four Sylvania germicidal lamps (radiation wavelength predominantly 2537 Å) placed 14 cm from the cell

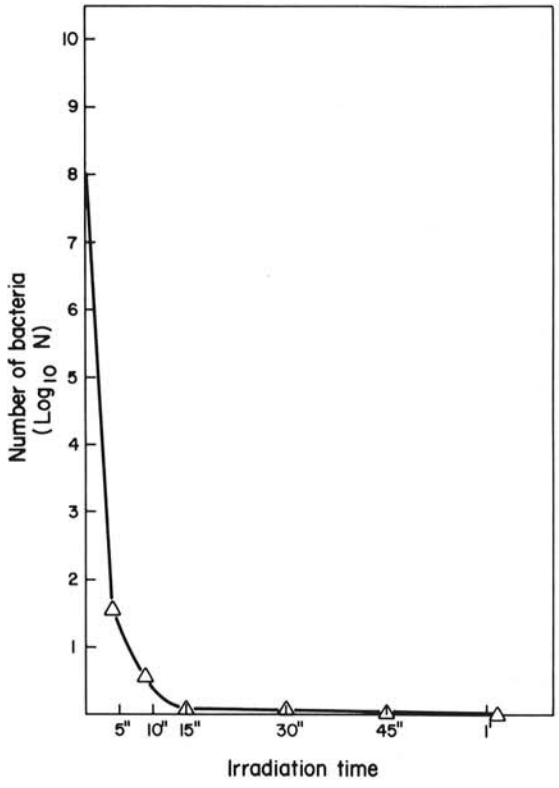

Fig. 1. Lethality curve of *Erwinia carotovora* to ultraviolet light.

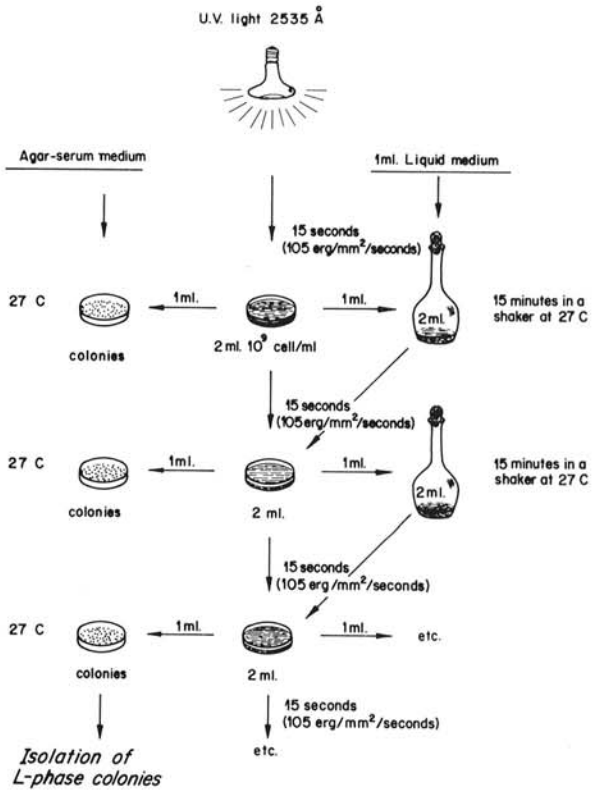

Fig. 2. Diagram of ultraviolet radiation procedure used to stimulate formation of L-forms of *Erwinia carotovora.*

suspension. The intensity of the four lamps totaled 105 erg/mm^2/sec. During irradiation, the cell suspensions were agitated by a shaker. After irradiation, the bacteria were grown on Koser 10% serum agar or in Koser 10% serum liquid medium.

Electron microscopy.—A suspension of L-forms *E. carotovora* in sterile distilled water was placed on a formvar-coated grid, and (following evaporation) shadowcasted with gold-palladium. Negative staining was done with buffered phosphotungstic acid, pH 6.8.

RESULTS

Lethality curve.—The bacterial suspensions were irradiated from 5 seconds to 1 minute. Following irradiation, 1-ml portions of the cell suspension were added to 100 ml of the buffer and portions of that suspension were appropriately diluted for plate counts.

After the lethality curve was obtained (Fig. 1) and the sensitivity of the bacteria to UV irradiation was established, it was found that the most suitable dose for the obtainment of the L-phase was 15 seconds.

Generation of L-colonies.—One milliliter of a suspension of the bacterial cells irradiated for 15 seconds was inoculated on Koser serum agar plates. In addition, 1 ml was diluted in 2 ml of liquid Koser medium and cultivated 15 minutes in a shaker at 25 C in a dark room. After this operation the liquid was placed in a sterile plate and irradiated again for 15 seconds (second irradiation).

The 1 ml cultivated on Koser serum agar plate was not irradiated; it was incubated so the growth characteristics could be observed at each stage of the process. From the twice-irradiated suspension, 1 ml was inoculated on Koser agar serum plate and 1 ml diluted again in liquid media and irradiated again (third irradiation).

After this procedure had been repeated five times white, dentate, 3-mm diameter colonies began to appear on the Koser serum agar plates. They were composed of spheroplasts, which have a nonrigid cell wall.

L-phase colonies were finally isolated by transfer from a single colony to a fresh serum agar plate (Fig. 2).

The L-phase colonies were clearly different from the normal-form colonies of *Erwinia carotovora,* which are larger, translucent, and have plain borders.

Inoculations in *Vicia faba* and *Phaseolus vulgaris* with L-phase colonies gave softening of the tissue and necrosis, but symptoms were less severe than those in the test inoculated with the normal parental *Erwinia carotovora.*

After five years of weekly transfer, these forms still conserve the same morphological and biological characteristics.

DISCUSSION

The L-form cells of *E. carotovora* are morphologically different from those obtained for other phytopathogenic bacteria by the penicillin technique. In the latter cells, only one envelope was observed (13), but the L-forms

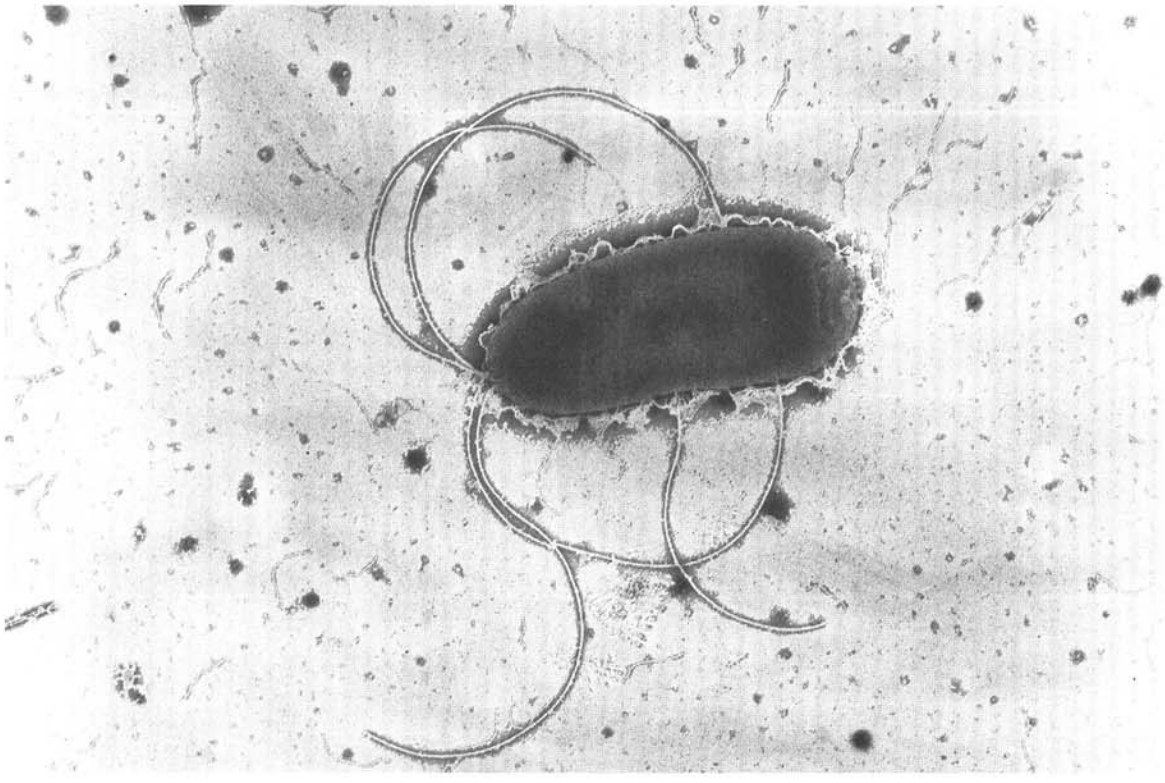

Fig. 3. *Erwinia carotovora* parental (nonirradiated) strain, negative staining. (× 15,000).

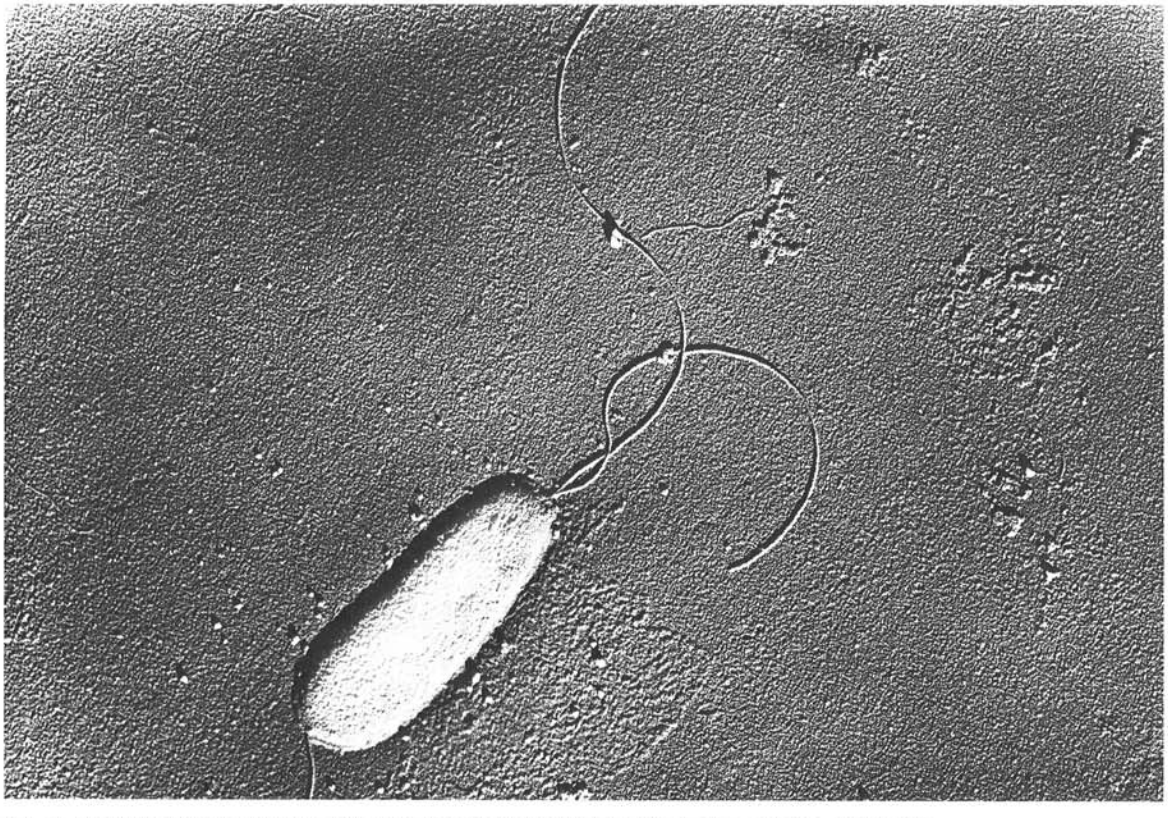

Fig. 4. *Erwinia carotovora* parental (nonirradiated) strain detailed with shadow-staining. (× 14,000).

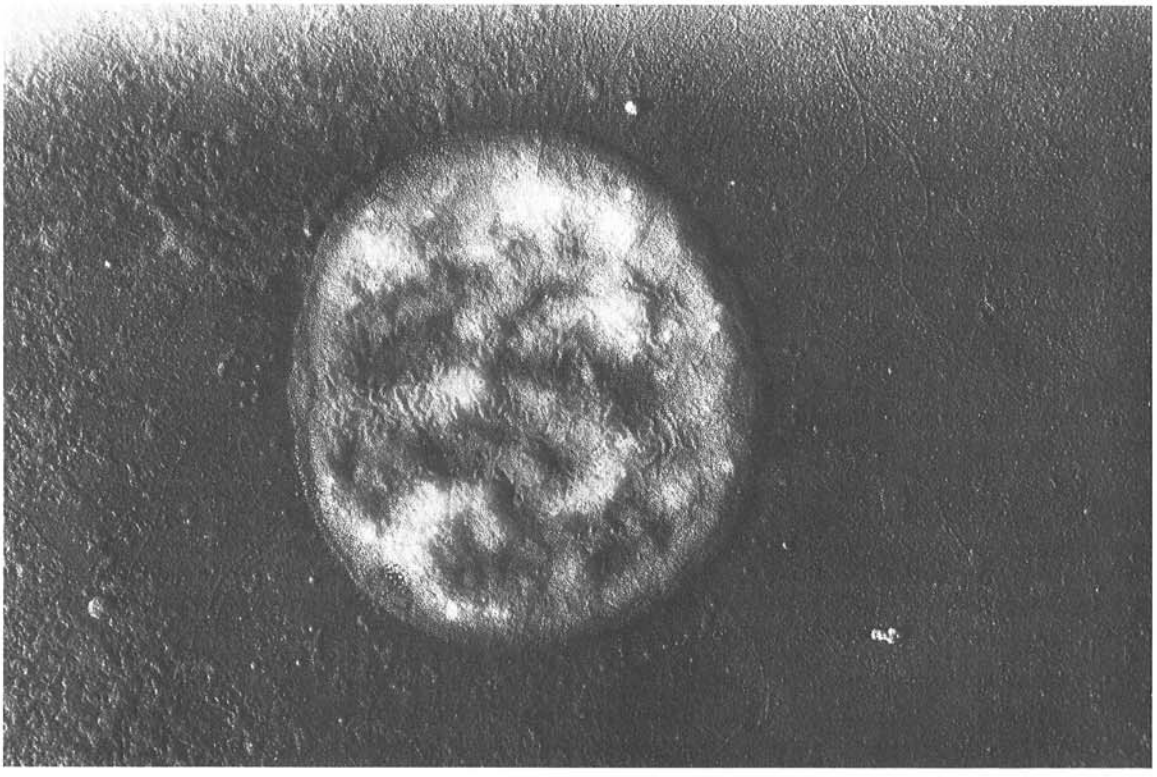

Fig. 5. L-form of *Erwinia carotovora* induced by ultraviolet light and stabilized with horse serum. (× 17,000).

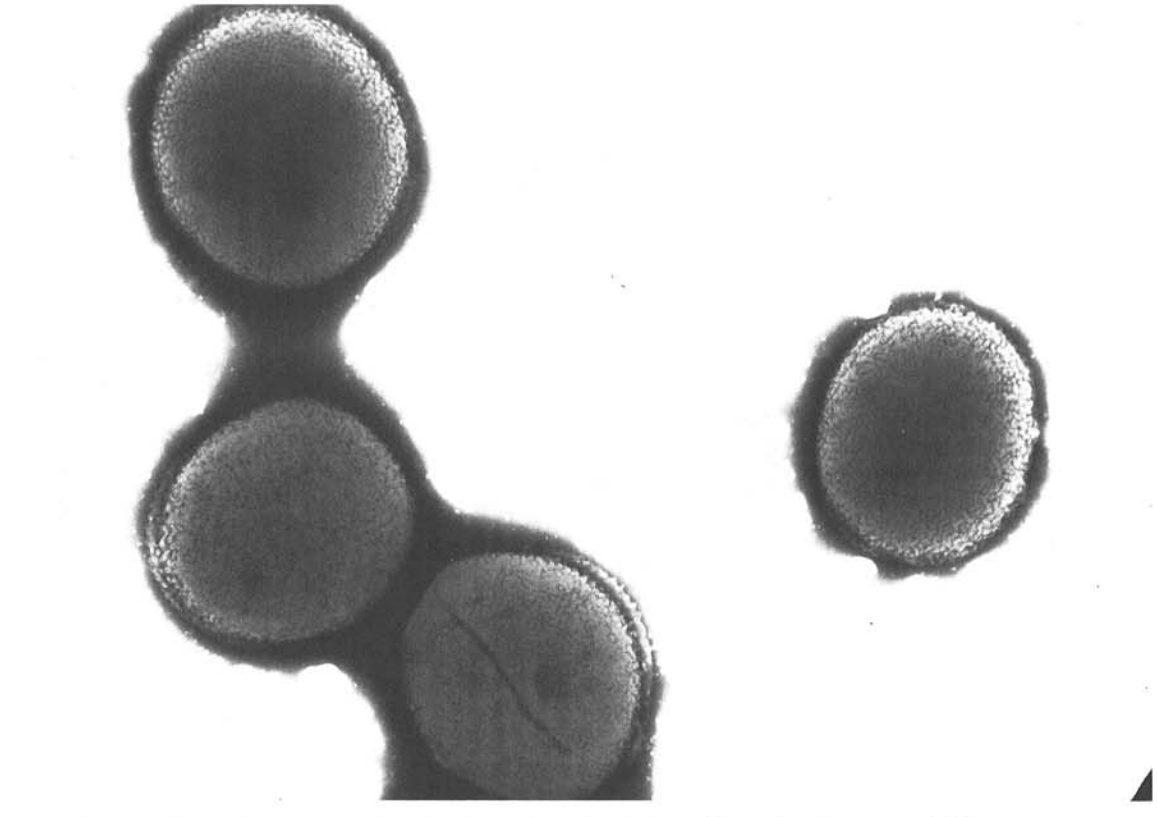

Fig. 6. L-form of *Erwinia carotovora* induced by ultraviolet light and stabilized with sucrose. (× 16,000).

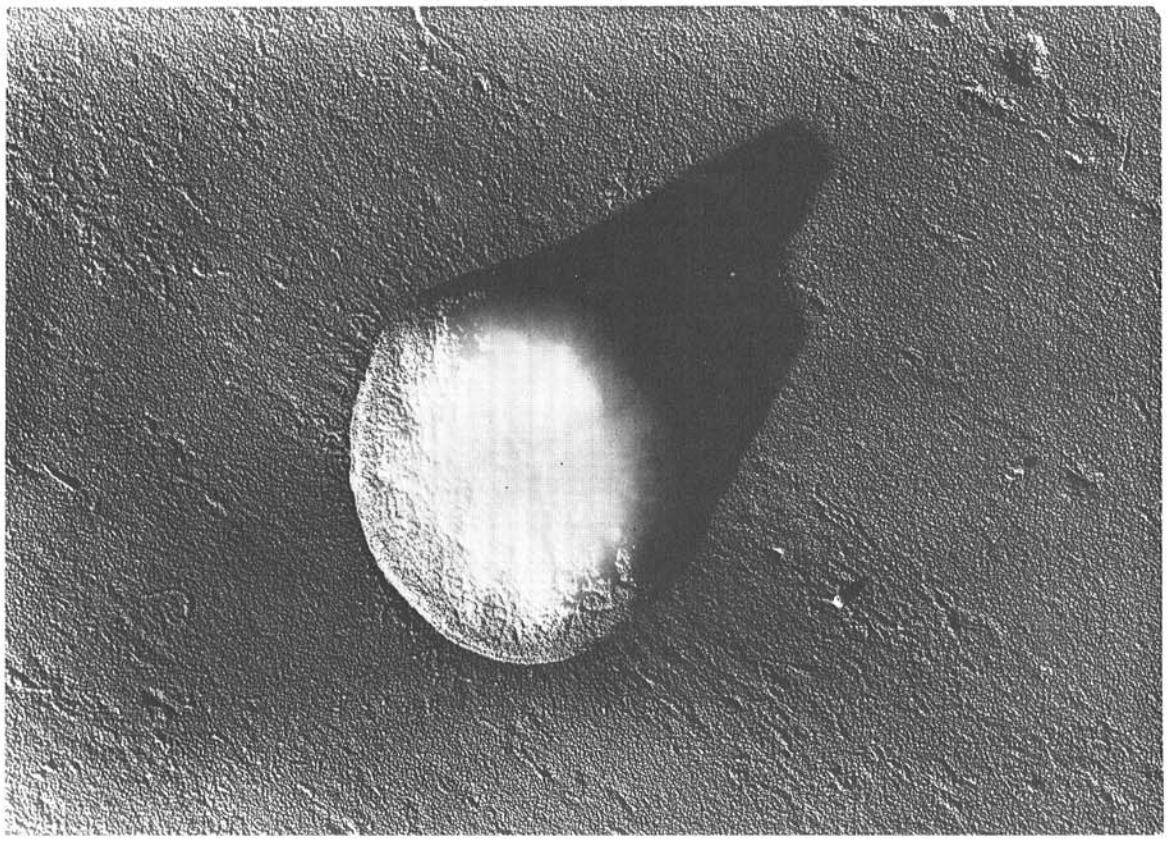

Fig. 7. L-form of *Erwinia carotovora* induced by ultraviolet light and stabilized with sucrose. (× 20,000).

obtained with ultraviolet light showed two well-differentiated layers, an outer degraded layer, and an inner or cytoplasmic membrane, similar to that obtained by the glycine technique in *A. tumefaciens* (10) (Fig. 3, 4, 5).

Horse serum seems to be necessary for the development and stability of the L-form of *E. carotovora*. In some cases in which horse serum was not added to the medium and 0.3 M sucrose was used, the cells retained the L-form, but the cell size became smaller, and the cells were more compact (Fig. 6, 7).

On the other hand, we attempted to obtain L-forms with the aid of horse serum only, without radiation, but after two months of daily transfers in this medium, we did not obtain modification of the bacillary form. Therefore, we were sure that L-forms were not induced by the serum.

Most L-phase cells of some animal and human pathogenic bacteria obtained by the penicillin technique seem to become nonpathogenic or less virulent, whereas those induced by glycine retain pathogenicity and toxin-producing properties (3, 10, 12).

Also the L-forms induced by glycine have two envelopes; one of them is a rest of cell wall which is plastic and has a different chemical composition than that of the parental form (14, 15). Therefore it seems that the L-phase cells induced by UV light have more properties in common with the ones induced by glycine than with those induced by penicillin.

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