

Yersinia pestis: Correlation of Ultrastructure and Immunological Status

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Ultrastructural identification and localization of the fraction 1 "envelope" antigen in the plague bacillus *Yersinia pestis* were the primary objectives of this brief study. The antigenicity of extra-cellular material between the bacilli in undisturbed cultured colonies and that of the pathogen per se were measured and correlated by means of the semi quantitative complement fixation method after incubation for 72 h at 37 C. When the amount of extracellular substance in wild-type T1 (virulent) bacteria was compared by electron microscopy with that in avirulent strains of *Y. pestis*, with and without passage through guinea pigs, we found that the material of interest was greatly attenuated or even absent in colonies that had not been passed through animals, whereas passage markedly augmented production of the material. We also explored the requirement for larger quantities of Ca^{2+} and Mg^{2+} in the culture media and discovered that without these ions production of the extracellular material was limited. These observations support the hypothesis that this extracellular substance between cultured *Y. pestis* bacilli of various strains represents the source of the fraction 1 envelope antigen.

It has been well documented that the fraction 1 "envelope" antigen of *Yersinia pestis* is the major cofactor for immunizing man against plague infection (1, 4, 10, 20). The use of highly purified, specific fraction 1 antigen for complement fixation (CF) and for passive hemagglutination tests is a fundamental practice in plague immunoserology and prophylaxis studies (5-7). The need for analyzing the ultrastructure of the bacillus as it relates to the degree of immunity is therefore apparent.

Crocker et al. (8), Golubinsky et al. (14), and Katz (18) have all investigated the fine structure of *Y. pestis*. Crocker observed sharply outlined bodies of high electron density embedded in a substance of low electron density; the latter material comprises the envelope (fraction 1) antigen. Ten years later, Katz reported that most of the envelope material was lost during preparation procedures—its remnants appearing as irregularly scattered, fibrillar matter. By electron microscopy, Golubinsky found that the morphology of *Y. pestis* was basically similar to that of other gram-negative microorganisms.

The threefold intent of the experiments reported here was to trace the development of the fraction 1 envelope antigen ultrastructurally in both virulent and avirulent strains of *Y. pestis* grown for varying periods of incubation at 37 C, to study the effects of certain chemical agents

on the production of the envelope antigen, and to determine the consequences of passage through animals on the yield of the pathogen's fraction 1 antigen. Another objective was to evaluate fraction 1 production by the semiquantitative determination method to correlate the amount of antigen with our ultrastructural findings.

MATERIALS AND METHODS

Strains. The following strains of *Y. pestis* were used in this study: wild-type T1, EV 76 (Paris) F, EV 76 (Paris) R, EV 406, and M 23. Wild-type T1, isolated from Tanzania, was kindly supplied by R. J. Henderson, Public Health Laboratory, Royal Infirmary, Worcester, England. New varieties of the original avirulent strain EV 76 (Paris), EV 76 (Paris) F and EV 76 (Paris) R, have undergone 6 and 18 passages, respectively, through guinea pigs previously inoculated with nontoxic dosages of iron sulfate (20 μ g per g of body weight) (16, 19). These passage cultures of EV isolates fulfill the criteria for residual virulence and retain the genetic markers typical for attenuated *Y. pestis* (3, 23). The EV 406 strain (19) was initially given to the late Karl F. Meyer by A. Dodin of the Pasteur Institute, Tananarive, Madagascar. M 23 (2), fully virulent in mice but reduced in virulence in guinea pigs, served as a nonenvelope control isolate.

Procedures. The microorganisms were stored in the lyophilized state, with selected working cultures transferred to blood agar slants (Difco blood agar base

+ 3% sheep blood). For ultrastructural studies, whole colonies were grown on the surfaces of blood agar or heart infusion agar (HIA) plates. For semiquantitative (7) determination of the envelope fraction 1 content, acetone-killed and dried plague bacilli were grown on HIA plates with the addition of 0.003 M CaCl_2 , 0.02 MgCl_2 , and 0.2% xylose (24). All cultures were maintained at 37 C and were sampled after 24, 48, and 72 h of growth.

The colonies used for electron microscopic examination were fixed *in situ* on the agar culture medium to avoid disturbing cell-to-cell relationships. Specimens were initially fixed in 4% glutaraldehyde buffered in 0.1 M cacodylate (pH 7.3) to which 0.1% ruthenium red had been added. This stain is necessary for the fixation of the fraction 1 cell envelope antigen and affords improved contrast and general ultrastructural preservation compared with Kellenberger's fixative (15). After a 20-min rinse in the ruthenium red buffer solution, the blocks were postfixed for 1 h in 1% osmium tetroxide (OsO_4) in this same solution. After two 20-min rinses in distilled water, the material was stained *en bloc* in saturated (aqueous) uranyl acetate at room temperature for 2 h or overnight at 4 C. Next, the blocks were dehydrated in graded ethanols and embedded in Spurr low-viscosity resin mixture. Ultrathin sections were cut with glass knives on a Porter-Blum MT-2 microtome. The sections were then stained with ethanolic uranyl acetate and lead citrate and subsequently examined in a Siemens 1A electron microscope.

For the semiquantitative reverse CF test (7), acetone-killed and dried 72-h-grown organisms were extracted in saline (10 mg/ml) for 24 h and centrifuged. The supernatant was then decanted for use, and CF tests were performed with 0.2-ml dilutions of a specific anti-fraction 1 serum (diluted 1:64 and previously determined as four combining units) and of the supernatant.

RESULTS

Ultrastructural studies. As anticipated (4, 10), virulent strains yielded more envelope antigen than avirulent strains, with the exception of the attenuated EV series, expressly passed through guinea pigs first injected with nontoxic dosages of iron sulfate to enhance both the immunogenicity and the virulence of the strain (16, 19). In this report, we make no attempt to discuss enhancement of virulence, but rather explore the major immunogenic antigen of fraction 1 demonstrated by the ultrastructure of *Y. pestis* after passage through animals.

The cellular morphology of several strains of *Y. pestis* can be seen in Fig. 1. In all strains, the ultrastructure was similar. Visualized by electron microscopy, *Y. pestis* closely resembled other well-preserved, gram-negative bacteria (21), but its morphology differed distinctly from that described in earlier published works (14, 15, 18). A densely staining, tightly adherent cell surface coat was consistently observed with this particular fixation (Fig. 1) and appeared to be

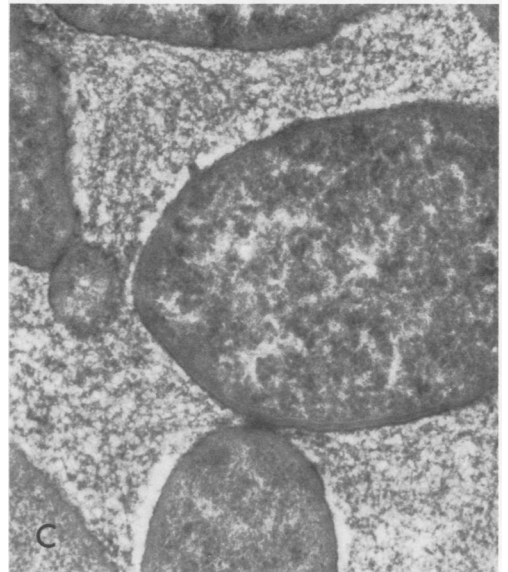
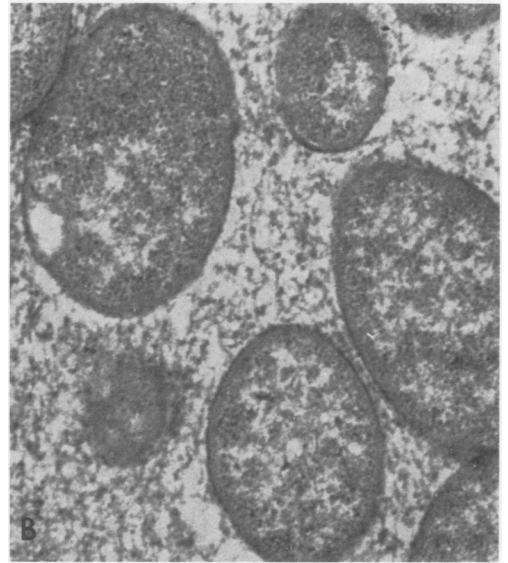
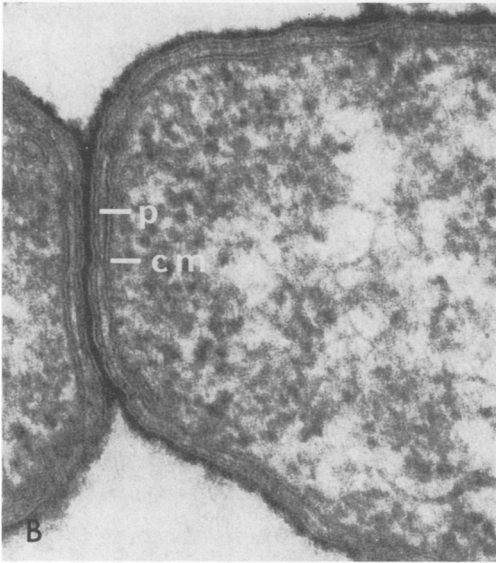
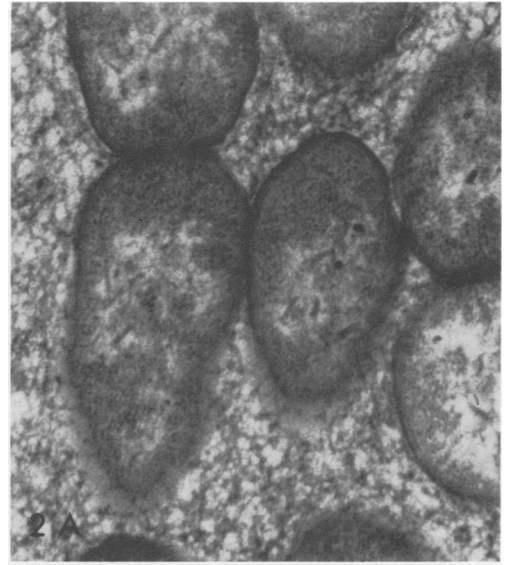
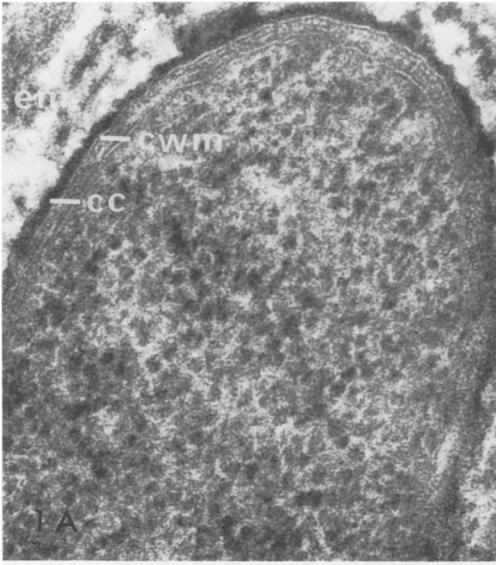
only slightly affected by uranyl acetate *en bloc* staining (not shown here).

Evidently ruthenium red prevents the deleterious effects of *en bloc* staining commonly observed (21). The next layer, proceeding inward, is the cell wall membrane, followed by a distinct R or peptidoglycan layer. Like the cell surface coat, this last layer appeared to be little affected by *en bloc* staining. The bacterial cell wall closely followed the contours of the cell membrane, with an even space imposed between the two layers (in contrast to the irregular cell wall noted with other fixatives [14, 15] and embedding media [14]). The internal cellular organelles were typical of the usual gram-negative organism. With this type of fixation, however, the nucleoid area was diffuse and undivided from the ribosome-rich region. Depending on the strain and the age of different colonies, the cells may be seen enmeshed in a fibrous or floccular envelope material. Ruthenium red proved to be a better fixative for this material than OsO_4 and glutaraldehyde alone or in combination with lanthanum and/or alcian blue.

After a 24-h incubation at 37 C, the *Y. pestis* grown on blood agar or HIA plates formed colonies of pinpoint-sized cells that reached normal size after 72 h of growth. Cells of the virulent strain T1, after incubation for 24, 48, or 72 h, were individually snared in an extensive, extracellular, fibrillar matrix resembling an envelope antigen (Fig. 2). In the case of the avirulent EV series [e.g., EV 76 (Paris) R], the extracellular matrix appeared only after 48 or 72 h of incubation (Fig. 3). This strain was grown on HIA plates with or without Ca^{2+} and Mg^{2+} (compare Fig. 4A and B with 4C and D). These results are summarized in Table 1.

The immunogenicity of strain EV 406 was poor as tested by the late Karl F. Meyer, who challenged activity immunized animals with a normally virulent strain (195/P) (unpublished data). Ultrathin sections revealed little if any extracellular material after 24 and 72 h of growth (Fig. 5A and B). However, when this strain was passed once through guinea pigs and then grown under the same conditions, after 72 h (but not after 24 h) of growth, it disclosed as much or more extracellular material as the virulent strain T1 (compare Fig. 5C and D with Fig. 2A-C). These findings are in accord with the results of the serological test at 72 h of growth before and after passage through animals (see Table 2).

The fraction 1-negative, mutant strain M 23 (2), treated identically, was compared with the other strains studied. There was no extracellular material in the 48-h colonies and very little in the 72-h ones (Fig. 6), thus confirming the



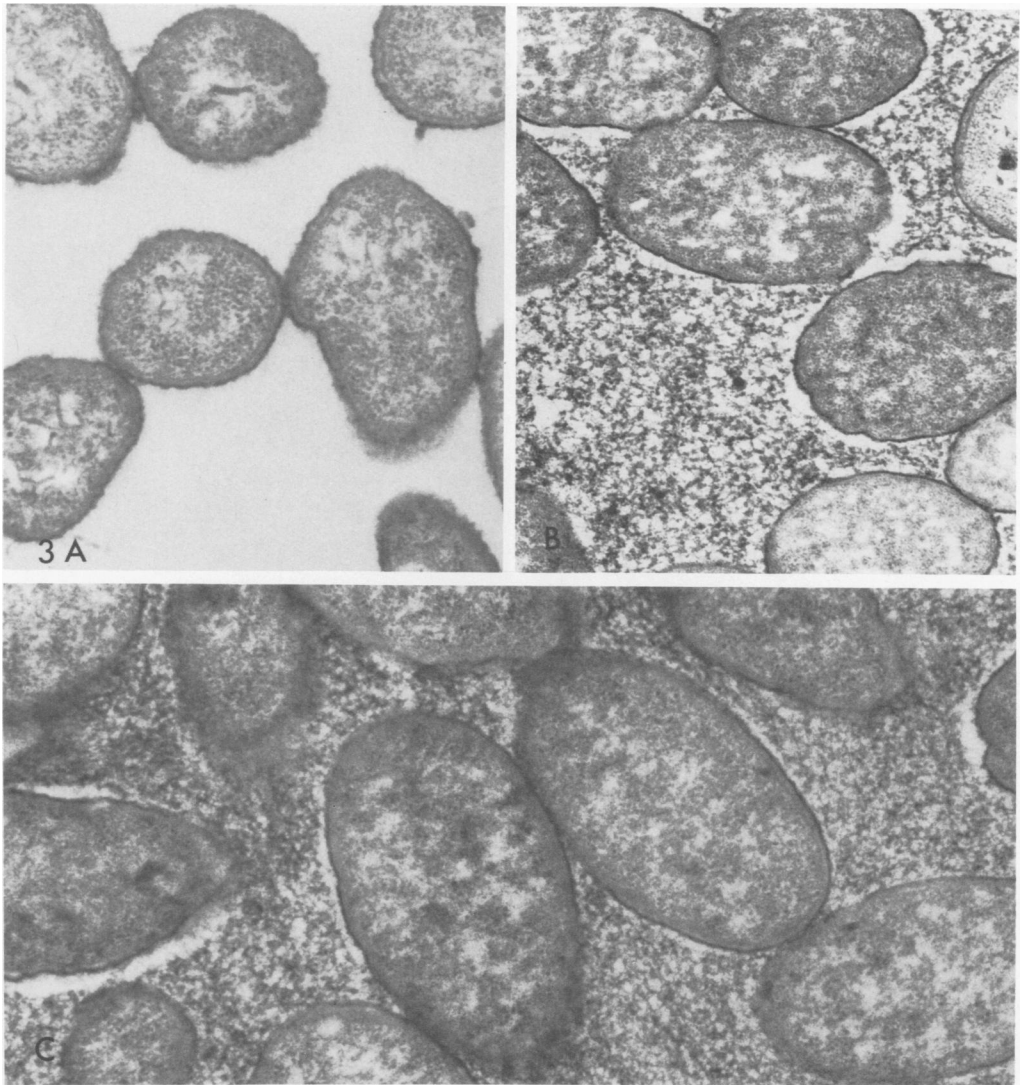


FIG. 3. Avirulent strain EV 76 (Paris) R grown on blood agar. The lack of extracellular material after 24 h of growth is obvious. (A) 24-h growth; $\times 10,000$. (B) 48-h growth; $\times 10,000$. (C) 72-h growth; $\times 10,000$.

FIG. 1. (A) Virulent *Y. pestis* strain T1 grown on blood agar for 72 h; $\times 35,000$. (B) Strain EV 76 (Paris) R grown on blood agar for 24 h; $\times 30,000$. (C) Strain M 23 grown on blood agar for 24 h; $\times 28,000$. Abbreviations: Cell surface coat, cc; cell membrane, cm; cell wall membrane, cwm; extra-cellular matrix or envelope, em; peptidoglycan or R layer, p.

FIG. 2. Virulent *Y. pestis* T1 grown on blood agar for: (A) 24 h ($\times 10,000$); (B) 48 h ($\times 11,000$); (C) 72 h ($\times 11,000$).

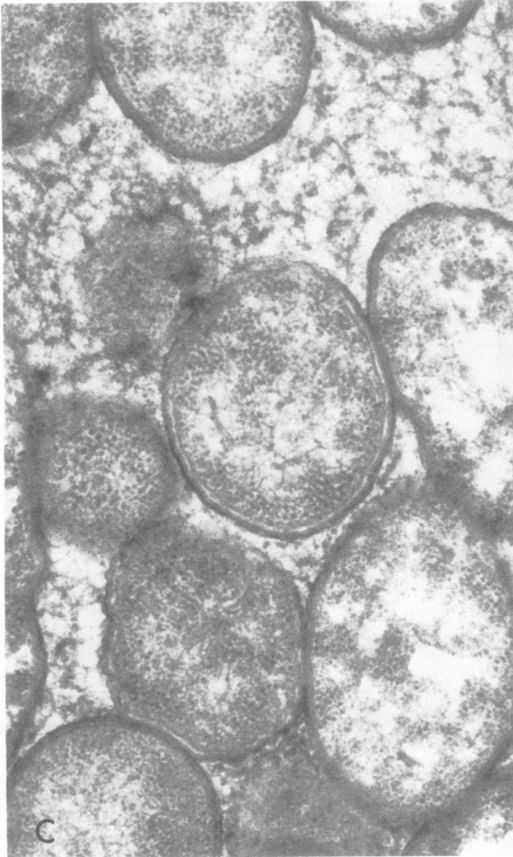
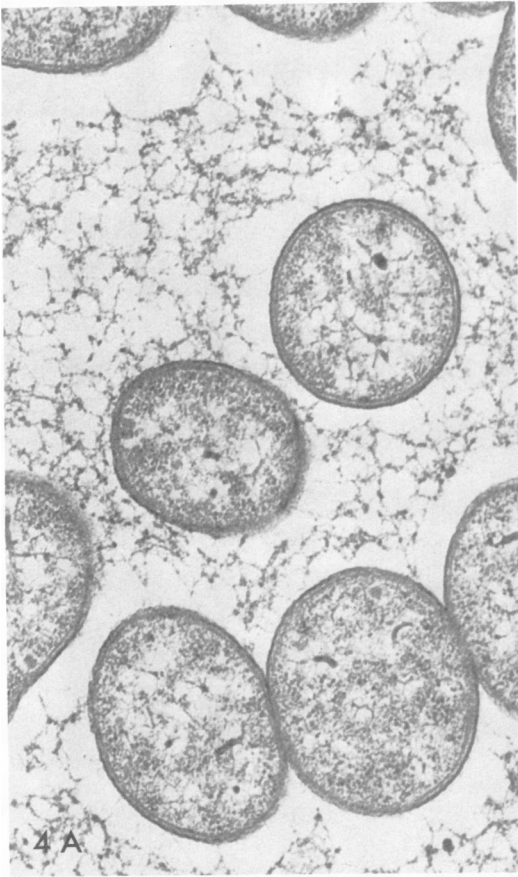


TABLE 1. Envelope (fraction 1) component induced by strains of *Y. pestis* under various experimental conditions: electron microscopic findings

Strain of <i>Y. pestis</i>	Culture medium	Duration of incubation (h) ^a	Envelope antigen induced ^b
Wild-type T1	Blood agar	24	+
		48	+
		72	+
EV 76 (Paris) R	Blood agar	24	-
		48	+
		72	+
EV 76 (Paris) R	HIA (no blood serum)	48	less
		72	less
EV 76 (Paris) R	HIA with Ca ²⁺ , Mg ²⁺ (no blood serum)	48	+
		72	+
EV 406	Blood agar	24	-
		72	-
EV406, one passage	Blood agar	24	-
		72	+
M 23	Blood agar	48	-
		72	-

^a All cultures were incubated at 37 C.

^b +, Fraction 1 positive; -, fraction 1 negative; less, less amount fraction 1.

earlier observation that M 23 is "nonenveloped" (2).

Assay of envelope antigen content. The fixation reactions of extracts of the 72-h culture of EV 76 (Paris) R, diluted as much as 1:2,048, were strongly positive, and the EV 76 (Paris) F extract yielded somewhat lower titers (1:1,024) (Table 2). Although EV 406 extracts reacted positively both before and after one passage through guinea pigs (in dilutions of 1:128 and 1:2,048, respectively), these results indicate that passage through animals is essential to the production of envelope antigen.

DISCUSSION

It is well established that the component constituting the envelope that surrounds the plague organism is the major immunity-inducing antigen against human plague infection (1, 4, 10, 20). In electron micrographs of undisturbed colonies, the extracellular material is clearly present after 24 h of growth in virulent-strain cultures but not in the immunogenically avirulent strain EV 76 (Paris) R. The material did appear in EV 76 (Paris) R, however, after 48 h of culture, as well as in once-passaged EV 406—suggesting that virulent and avirulent strains differ only in the time required to

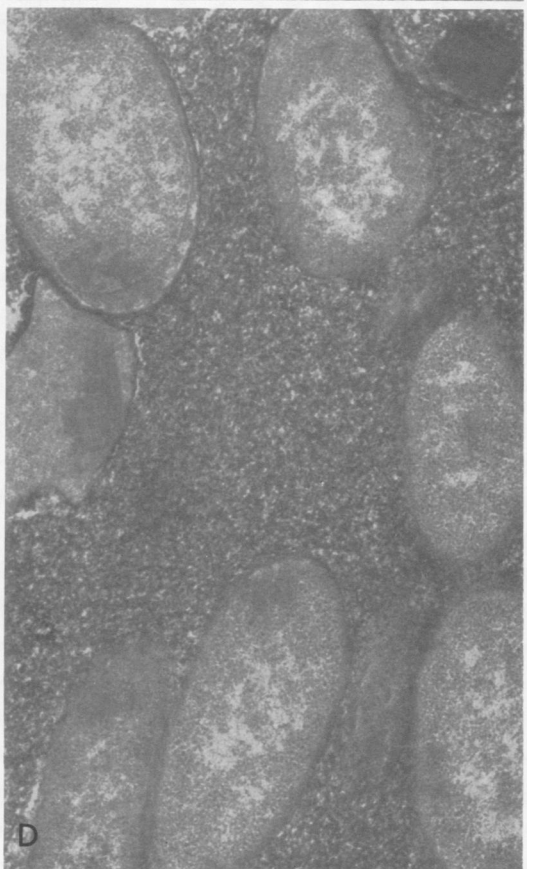
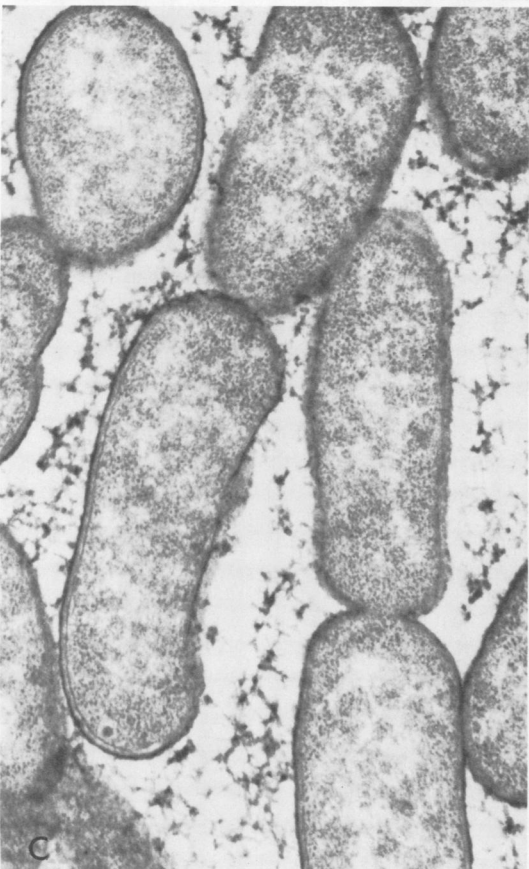
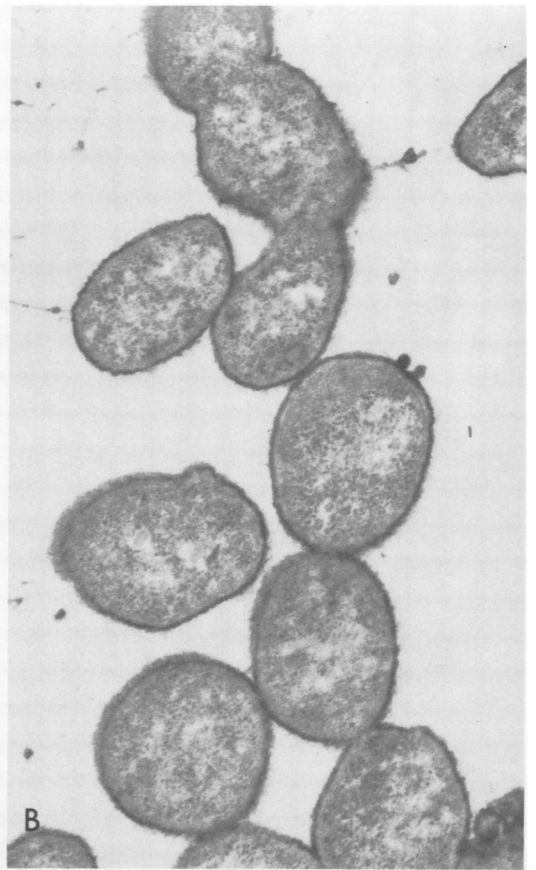
produce the envelope material. This factor is probably related to the enhanced sensitivity of avirulent organisms to phagocytosis during the first 24 h of growth, since the presence of the envelope is associated with resistance to phagocytosis (5, 17). Moreover, the fully virulent enveloped plague bacillus has proven capable of establishing lethal infection in all animals after intradermal injection with even a dosage approaching that of a single bacterium (9).

The passage of *Y. pestis* through an animal (guinea pig) restores the original virulence of the pathogen. In 1965, Jackson and Burrows (16) achieved enhancement of virulence by injecting low-virulence strains of *Y. pestis* into animals treated with nontoxic doses of iron salts. The EV 76 (Paris) F (six passages) and EV 76 (Paris) R (18 passages) were obtained in this manner (19). As documented in Table 2, the passaged R strain contains more fraction 1 than the passaged F isolate. It is of greater significance, however, that strain EV 406, passed once through animals without the previous injection of iron salts, exhibited more extracellular material and higher CF titers than before passage. This finding supports the theory that passage through animals selectively heightens the immunogenicity of the "more antigenic variants."

Gadgil et al., in 1966 and 1967 (11-13), demonstrated that the absence of Ca²⁺ in the culture medium caused considerable pleomorphism: after 24 h, the organisms were either thin and small, enlarged, or unduly elongated. With the addition of Ca²⁺ and Mg²⁺ to the medium, virulent *Y. pestis*, grown at 37 C, acquired more antigenic components. This observation was further supported by estimating the total protein in the cultures as well as by performing bacterial counts. In 1974, Hall et al. (15) indicated that at 37 C continuous exposure to Ca²⁺ is imperative if the bacilli are to retain their normal morphology and ability to divide. Our EV 76 (Paris) R, grown on HIA plates with and without Ca²⁺ and Mg²⁺, again emphasized the need for these ions in the production of extracellular material. Thus, the addition of the two ions to the medium is indisputably essential in preparing antigenic cultures of *Yersiniae* for the immunoprophylaxis of plague.

Semiquantitative determination of fraction 1 content in the supernatant fluid by means of the CF test is not as accurate as the methods used by Baker et al. (1) and by Silverman et al. (22), but simply for purposes of comparing amounts

FIG. 4. Avirulent *Y. pestis* EV 76 (Paris) R strain grown on HIA (no blood serum) with and without Ca²⁺ and Mg²⁺ (A) 48-h growth without added ions; $\times 9,000$. (B) 72-h growth without ions; $\times 10,000$. (C) 48-h growth with added ions; $\times 14,000$. (D) 72-h growth with added ions; $\times 9,000$.



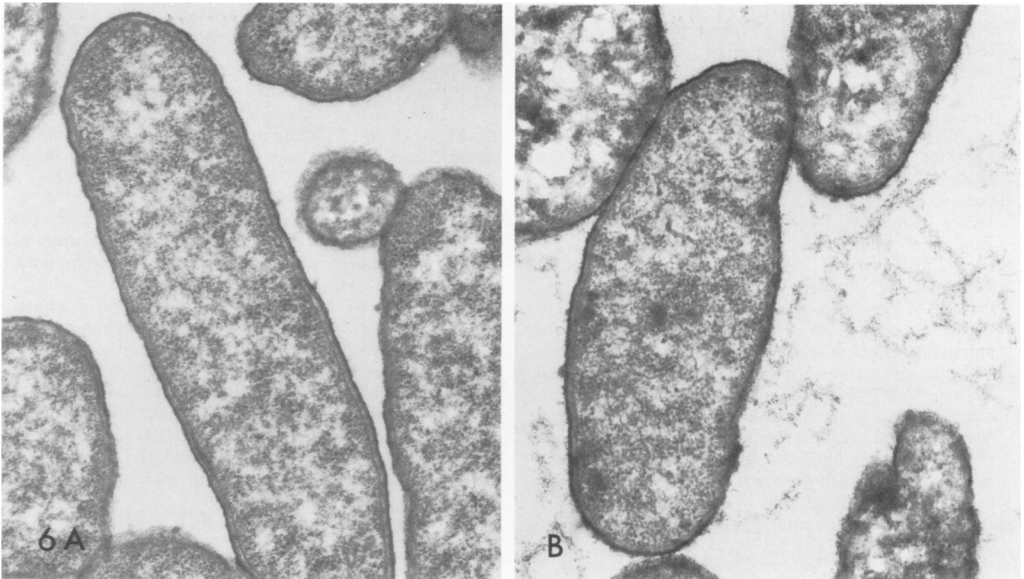


FIG. 6. Nonenvelope strain M 23 grown on blood agar for (A) 48 h ($\times 10,000$) and (B) 72 h ($\times 11,000$).

TABLE 2. CF determination of the fraction 1 content in strains of *Y. pestis*

Strain	CF titer ^a
EV 76 (Paris) R	1:2,048
EV 76 (Paris) F	1:1,024
EV 406	1:256
EV 406 (one passage)	1:2,048
Fraction 1 (1 mg/ml)	1:2,048

^a Reciprocal of dilution of supernatant fluid.

of antigen, the procedure is adequate. Indeed, the results we obtained with this test substantiated our ultrastructural evidence and reinforced our claim that the extracellular material observed in thin sections of *Y. pestis* is the source of the "envelope antigen."

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FIG. 5. Avirulent *Y. pestis* EV 406 grown on blood agar for (A) 24 h, without animal passage ($\times 10,000$); (B) 72 h, without passage through animal ($\times 9,000$); (C) 24 h, with one passage ($\times 10,000$); (D) 72 h, with one passage ($\times 10,000$).

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