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Localization of Enterobacterial Common Antigen: *Proteus mirabilis* and Its Various L-Forms

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An investigation of *Proteus mirabilis* wild-type strains and their various derived L-forms shows that the enterobacterial common antigen (ECA) is localized in the outer membrane of the cell envelope of these strains. In strains where the outer membrane is lacking (stable protoplast L-forms) or where its amount is reduced (spheroplast UL19) no ECA or only reduced amounts of it are detected by serological tests or by ferritin-labeling techniques.

In the preceding paper (19), it was shown that the enterobacterial common antigen (ECA) is readily accessible for specific antibodies in whole cells of *Escherichia coli*. The conclusion is therefore inevitable that ECA is localized in the outer membrane of this organism. On the other hand, it was found by Domingue and Johnson (1) and Johnson et al. (6) that ECA specificity was distributed between the outer and the cytoplasmic membrane after fractionation of the *E. coli* cell envelope. However, ECA is an amphophilic molecule (10, 16, 23) showing a high affinity for membranes, and these authors could not exclude the possibility that ECA had been redistributed between both membranes during the fractionation procedure (1, 2). In this paper, *Proteus mirabilis* wild type and L-forms are examined for the presence of ECA to investigate whether ECA is localized solely in the outer membrane or distributed also in the cytoplasmic membrane or the cytoplasm.

P. mirabilis is able to survive in the presence of high concentrations of penicillin and to multiply as the unstable L-form. These cells are fragile spheroplasts with a defective cell wall which still synthesize outer membrane and peptidoglycan (5, 7, 14). A second type of L-form, the stable protoplast L-form, has completely lost its cell wall. The sole remaining surface layer is the morphological equivalent of the bacterial cytoplasmic membrane. Neither peptidoglycan nor major outer membrane proteins can be detected, but small amounts of lipopolysaccharide are still retained in the protoplast membrane (3, 4, 7, 13). Therefore, the two types of L-forms seemed to be well-suited organisms with which to investigate the problem of ECA localization. The results reported in this paper (i.e., ECA present in the *P. mirabilis* wild-type and unstable spheroplast L-form, but no ECA detectable in the stable protoplast L-form) strongly indicate that ECA is exclusively located in the outer membrane of the enterobacterial cell.

MATERIALS AND METHODS

Chemicals. Ferritin-conjugated and fluoresceine-labeled goat anti-rabbit immunoglobulin G (IgG) were obtained from Miles, Elkhart, Ind.

Bacterial strains. Stable protoplast L-forms LD52, LVI and the unstable L-form spheroplast UL19 were obtained from *P. mirabilis* wild-type strains D52, VI, and 19 by penicillin treatment as described by Gmeiner et al. (3) and Martin and Gmeiner (14).

Antisera. Antisera were obtained by intravenous immunization of New Zealand White rabbits. Rabbits were immunized three times at 4-day intervals with increasing amounts of a living suspension of the immunogenic ECA strain *E. coli* F470 (15, 20). The resulting antisera were absorbed with the ECA-negative mutant *E. coli* F1283 as described in the preceding paper (20) and were then separated on a Sephadex G-200 column into the IgM and IgG antibody fraction (20).

Serological methods. Passive hemagglutination and its inhibition were performed with human erythrocytes (bloodgroup A) as described in detail by Neter (17) and Männel and Mayer (12). Briefly, 25 μl of a dilution of cell extract containing from 250 to 1 μg of material was added to 25 μl of an appropriate serum dilution (3 hemagglutination units). After incubation for 1 h at 37°C, 50 μl of a suspension of sensitized erythrocytes was added, and the microtiter plates were incubated again for the same time. The lowest concentration of cell extract still giving a total inhibition of the hemagglutination reaction was determined by visual examination.

Immunoelectrophoresis was carried out by the microtechnique of Scheidegger (21) with an electrophoresis chamber from Gelman Instrument Co. (model 51170-1) and a sodium barbiturate buffer of pH 8.6 at a field strength of 10 V/cm for 1 h.

Labeling with ferritin. For the labeling and control experiments, the indirect labeling procedure with isolated IgG obtained from an absorbed ECA antiserum was used as described recently in detail by Geyer et al. (2) and in the preceding paper (19).

Electron microscopy. The freeze-etch preparation and the electron microscopical examination of labeled bacteria were performed as described in the preceding paper (19).

For thin sectioning, labeled bacterial cells were fixed for 2 h in 2% glutardialdehyde in 0.05 M cacodylate buffer of pH 7.1 (17). Fixed cells were washed in barbital-buffered saline, suspended in agar, dehydrated in ethanol (8), and embedded in Epon (9). Sections were mounted on copper grids coated with Formvar and were stained with uranyl acetate and lead citrate (19). These sections were also examined in a Philips EM 400 electron microscope.

Labeling with fluorescent antibodies. Bacteria labeled with the IgG fraction of an absorbed ECA antiserum were incubated with commercial fluorescent goat anti-rabbit IgG. Details of the procedure are given in the preceding paper (20).

RESULTS AND DISCUSSION

ECA content of bacterial strains. The ECA content of *P. mirabilis* strains (wild types, spheroplasts, and protoplasts) was determined by serological techniques with a high-titered precipitating ECA antiserum (12, 20). Supernatants of heated bacterial suspensions, or phenol-water extracts of bacteria, were used as sources of ECA for passive hemagglutination and for agar gel precipitation (Ouchterlony and electrophoresis). High ECA titers were obtained in passive hemagglutination with all three wild-type strains (Table 1) whether 100 μl or 1 ml of the supernatant was taken for erythocyte sensitization (for details, see legend to Table 1). The spheroplast UL19 showed a considerably lower hemagglutination titer, especially when the 100-μl amount was taken for erythrocyte coating.

In contrast, the two stable protoplast L-forms were found to lack ECA. When 1 ml of the supernatant from strain LVI was used for coating the erythrocytes, only a low hemagglutination titer was obtained. With the wild-type strain, 10 μl of the supernatant was enough to coat the erythrocytes and to obtain a hemagglutination titer of about 640. Similar results were obtained in the inhibition reaction, where extracts from the protoplast L-forms were noninhibiting. Surprisingly the spheroplast phenol-water extract contained a large amount of inhibitory substance. A similar finding was made by Whang and Neter (24) with glycine-induced spheroplasts of *Salmonella typhi*. In their experiments the supernatant of the suspension failed to modify erythrocytes for ECA agglutination, whereas the spheroplast suspension itself inhibited hemagglutination. Considering the heterogeneity of ECA (10-12), it is possible that not all ECA species are reduced to the same extent in spheroplasts.

The results in agar gel precipitation and immunoelectrophoresis are also depicted in Table 1 and indicate that phenol-water extracts from spheroplasts again show a reduced capacity to react with ECA antiserum and that extracts from protoplasts fail to react.

Ferritin-labeling studies. By using the indirect labeling technique, no adhering ferritin particles were seen in preparations of protoplast LD52 (Fig. 1), whereas good or moderate labeling was seen in the preparations of the wild-types (not shown) and of the spheroplast UL19. With spheroplast UL19 (Fig. 2), some areas with

TABLE 1. *Serological activity of extracts from P. mirabilis wild-type cells, sphero- and protoplasts with an ECA antiserum*

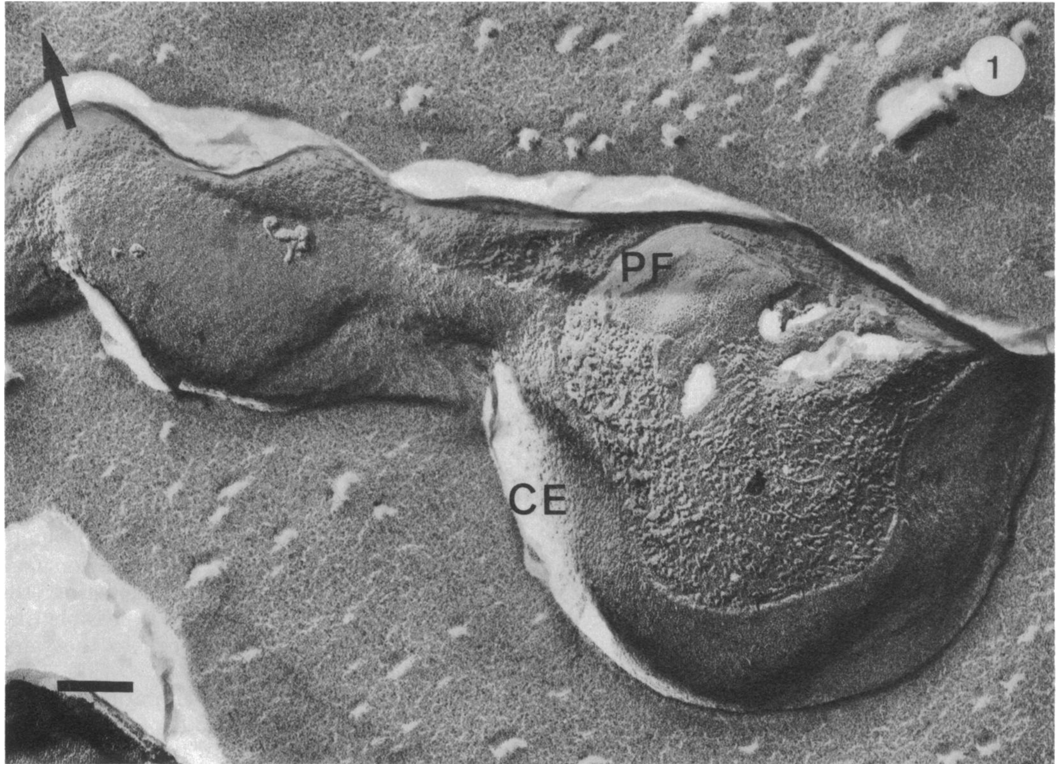
P. mirabilis strain	Titers in passive hemagglutination with:				Inhibition of passive hemagglutination (minimal inhibiting dose, μg/ml)[c]	Precipitation in agar-gel[d]
	Supernatant of heated culture[a]		Phenol-water extracts[b]			
	A	B	C	D		
Wild type						
D52	5.120	5.120	5.120	10.240	62.5	++
VI	5.120	5.120	5.120	10.240	62.5	++
19	5.120	5.120	2.560	5.120	62.5	++
Spheroplast						
UL19	320	1.280	160	1.280	62.5	+
Protoplast						
L D52	<10	<10	<10	<10	>250	−
L VI	<10	160	<10	<10	>250	−

[a] A 50-mg bacterial dry mass was heated (1 h, 100°C) in 5 ml of saline; 25 μl of erythrocyte sediment was incubated with (A) 100 μl or (B) 1 ml of the supernatant fraction.

[b] Erythrocytes (25 μl of packed cells in 5 ml of saline) were incubated with (C) 100 μg or (D) 400 μg of lyophilized aqueous phase material of phenol-water extracts from cells.

[c] Aqueous-phase material from phenol-water extracts was used as inhibitor, ECA from strain D52 was used as indicator antigen, and rabbit antiserum against *E. coli* F470 (20) was used as ECA antiserum.

[d] Lyophilized aqueous-phase material (0.1% in saline) was used for agar gel precipitation in Ouchterlony and immunoelectrophoresis.



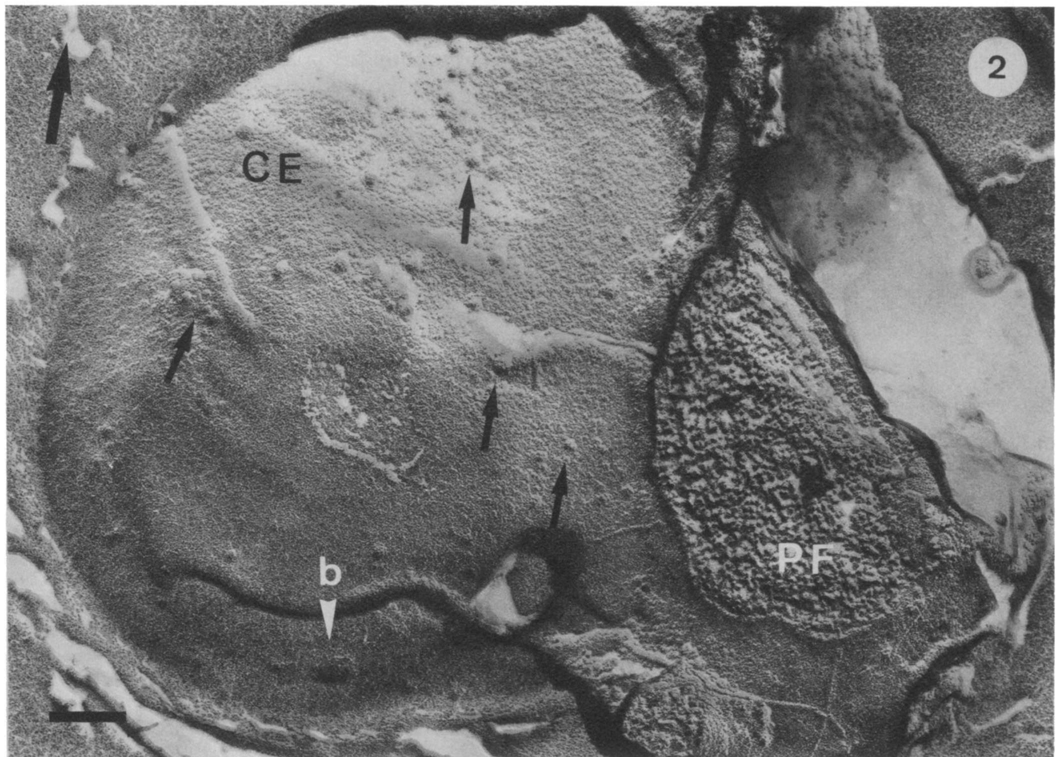
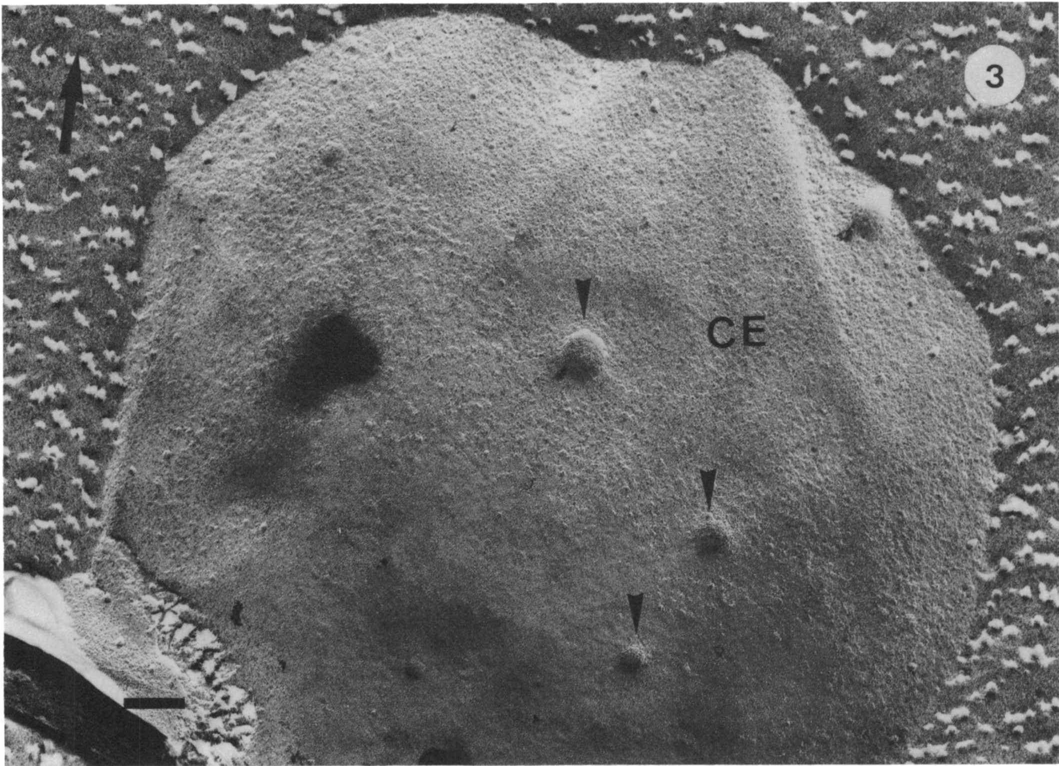


FIG. 1. *Freeze-etching of P. mirabilis protoplast LD52 after labeling by the indirect method (IgG from absorbed ECA antiserum; ferritin-conjugated goat anti-rabbit IgG). The outer fracture face of the cell envelope (CE) is completely devoid of ferritin particles. PF marks the inner fracture face of the cytoplasmic membrane, the arrow in the upper left corner indicates the direction of shadowing, the bar represents 100 nm; magnification is 100,000-fold.*

FIG. 2. *Freeze-etching of P. mirabilis spheroplast UL19 after labeling by the indirect method (for details, see legend to Fig. 1). The etched surface of the outer side of the cell envelope (CE) shows a number of ferritin particles (indicated by small arrows) and one bleb (b).*



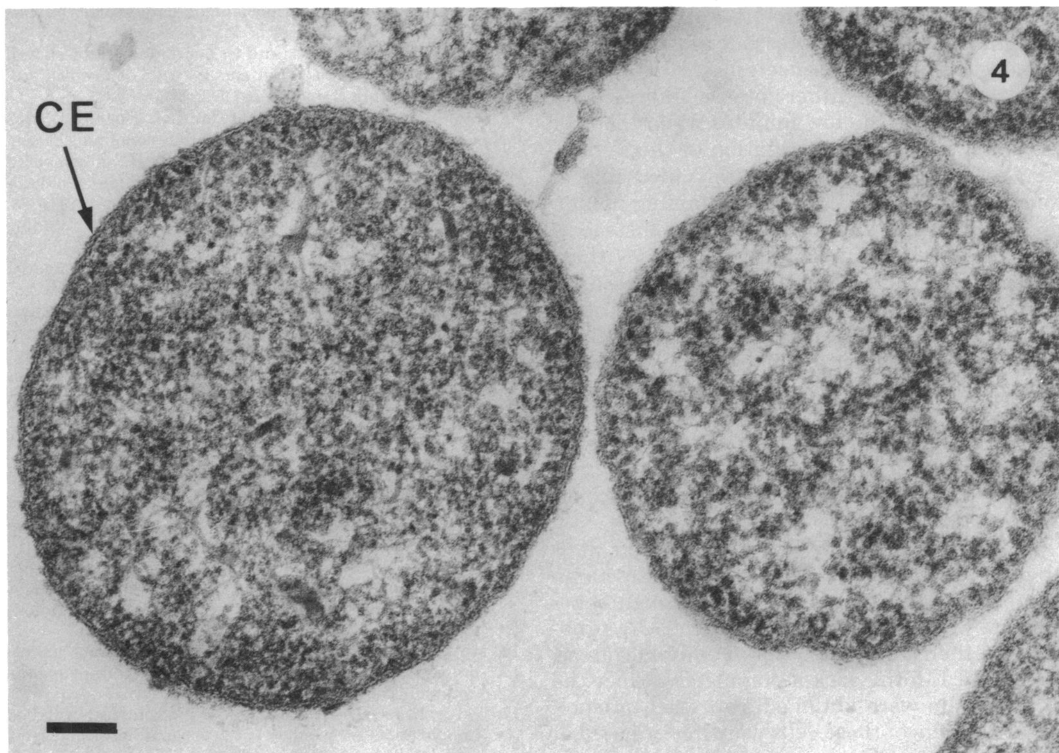


FIG. 3. *Freeze-etching of P. mirabilis spheroplast UL19 (negative control to Fig. 2) after labeling by the indirect method (IgG from serum of nonimmunized rabbit and ferritin-conjugated anti-rabbit IgG). The outer face of the cell envelope (CE) shows no ferritin particles. Some so-called blebs (b) are detectable at the surface of the cell. For details see text and legend to Fig. 1. The magnification is 80,000-fold.*

FIG. 4. *Ultrathin section of protoplast LD52 from P. mirabilis after labeling by the indirect method (IgG from absorbed ECA-antiserum and ferritin-conjugated goat anti-rabbit IgG). The cell envelope (CE) shows no ferritin particles. The bar represents 100 nm; magnification is 100,000-fold.*

ferritin label and some without ferritin particles were observable, probably reflecting the location of the residual outer membrane material.

In a control experiment the spheroplast UL19 was labeled by the indirect method with the IgG fraction of a normal rabbit serum and a commercial goat anti-rabbit IgG conjugated to ferritin (Fig. 3). The surface of the freeze-etched spheroplast has a smooth appearance without any ferritin particles adhered; however, some blebs (indicated as b in Fig. 2 and 3) are recognizable. These blebs were reported by Smit et al. (22) to represent vesicles of outer membrane material and to occur especially in enterobacterial R mutants (see also reference 20). Their presence in the spheroplast and absence in protoplast L-forms agree with the assumption that they contain outer membrane material.

These findings by ferritin-labeling agree with the above-discussed results by serological techniques.

Ultrathin sections. Ultrathin sections of labeled protoplast LD52 are shown in Fig. 4. No residual outer membrane material and no ferritin particles are recognizable on the outside of the cell envelope (CE).

Studies with fluorescent antibodies. Indirect labeling with fluorescent antibodies also demonstrated the presence of ECA. The spheroplasts showed a rather intense fluorescence, but only during the few minutes when the cells were still alive. The distribution of the fluorescent intensity was approximately the same as found recently (20) for ECA-containing nonimmunogenic *E. coli* strains. However, instead of the cell surface fluorescing, only two or three brightly stained areas were visible. These regions were not fixed but moved over the surface of the spheroplast, sometimes joining and sometimes separating again. Under irradiation with UV light, the spheroplasts UL19 were unstable, bursting in 2 to 5 min. No intact cells could be found in the sediment layer; the fluorescence was adhering to amorphous particles.

Protoplast L-forms showed no fluorescence when examined under the UV microscope.

From the results of this investigation, it is obvious that ECA is restricted to the outer membrane of the cell envelope. Even though spheroplasts were found to contain some ECA, probably in their residual outer membrane areas, protoplast L-forms lack it completely. Since the same results were obtained also when phenol-water extracts of these cells were investigated, it is evident that ECA is not present in the cytoplasm in larger than trace amounts. Thus, ECA presence is similar to that of the outer membrane proteins which are lacking in stable protoplast membrane, whereas lipopolysaccharide is at least partly integrated in the protoplast membrane (3, 4).

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LITERATURE CITED

1. **Domingue, G. J., and E. Johnson.** 1974 Isolation of subcellular fractions containing immunogenic enterobacterial common antigen. Z. Immunitätsforsch. **148:** 23–38.
2. **Geyer, R., C. Galanos, O. Westphal, and J. R. Golecki.** 1979 A lipopolysaccharide-binding cell-surface protein from *Salmonella minnesota*. Eur. J. Biochem. **98:**27–38.
3. **Gmeiner, J., H.-P. Kroll, and H. H. Martin.** 1977 Membrane composition of the cell-wall-less protoplast L-form of *Proteus mirabilis*, p. 175–184. *In* J. Roux (ed.), Spheroplasts, protoplasts and L-forms of bacteria. Editions INSERM, vol. 65, Paris.
4. **Gmeiner, J. and H. H. Martin.** 1976 Phospholipid and lipopolysaccharide in *Proteus mirabilis* and its stable protoplast L-form. Eur. J. Biochem. **67:**487–494.
5. **Hofschneider, P. H., and H. H. Martin.** 1968 Diversity of surface layers in L-forms of *Proteus mirabilis*. J. Gen. Microbiol. **51:**23–33.
6. **Johnson, E. J., G. J. Domingue, and G. D. Klein.** 1976 Anatomical locus of the common enterobacterial antigen. Z. Immunitätsforsch. **152:**15–26.
7. **Kandler, O., A. Hund, and C. Zehender.** 1958 Cell wall composition in bacterial and L-forms of *Proteus vulgaris*. Nature (London) **181:**572–573.
8. **Kellenberger, E., A. Ryter, and J. Sechaud.** 1958 Electron microscope study of DNA-containing plasm. II. Vegetative and mature phage DNA as compared with normal bacterial nucleotides in different physiological states. J. Biophys. Biochem. Cytol. **4:**671–683.
9. **Luft, H. J.** 1961 Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. **9:**409–414.
10. **Mäkelä, P. H., and H. Mayer.** 1976 Enterobacterial common antigen. Bacteriol. Rev. **40:**591–632.
11. **Männel, D., and H. Mayer.** 1978 Isolation and chemical characterization of the enterobacterial common antigen. Eur. J. Biochem. **86:**361–370.
12. **Männel, D., and H. Mayer.** 1978 Serological and immunological properties of isolated enterobacterial common antigen. Eur. J. Biochem. **86:**371–379.
13. **Martin, H. H.** 1964 Composition of the mucopolymer in cell walls of the unstable and stable L-forms of *Proteus mirabilis*. J. Gen. Microbiol. **36:**441–450.
14. **Martin, H. H., and J. Gmeiner.** 1979 Modification of peptidoglycan structure by penicillin action in cell walls of *Proteus mirabilis*. Eur. J. Biochem. **95:**487–495.
15. **Mayer, H., and G. Schmidt.** 1971 Hämagglutinine gegen ein gemeinsames Enterobacteriaceen-Antigen in *E. coli* R1-Antiseren. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1. Orig. Reihe A **216:**299–313.
16. **Mayer, H., and G. Schmidt.** 1979 Chemistry and biology of the enterobacterial common antigen. Curr. Top. Microbiol. Immunol. **85:**99–151.
17. **Neter, E.** 1965 Indirect bacterial hemagglutination and its application to the study of bacterial antigens and serological diagnosis. Pathol. Microbiol. (Basel) **28:**859–877.
18. **Reynolds, E. S.** 1963 The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17:**208–212.
19. **Rinno, J., J. R. Golecki, and H. Mayer.** 1980. Locali-

zation of enterobacterial common antigen: immunogenic and nonimmunogenic enterobacterial common antigen-containing *Escherichia coli* strains. J. Bacteriol. **141**:814-821.

20. **Sabatini, D. D., K. Bensch, and R. J. Barnett.** 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. **17**:19-58.
21. **Scheidegger, J. J.** 1955 Une micro-methode de l'immunoelectrophorese. Int. Arch. Allergy **7**:103-110.
22. **Smit, J., Y. Kamio, and H. Nikaido.** 1975 Outer membrane of *Salmonella typhimurium*: chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. J. Bacteriol. **124**:942-958.
23. **Whang, H. Y., and E. Neter.** 1962 Immunochemical studies of a heterogenetic enterobacterial common antigen (Kunin). J. Bacteriol. **84**:1245-1250.
24. **Whang, H. Y., and E. Neter.** 1965 Demonstration of common antigen in *Salmonella typhi* spheroplasts. Can. J. Microbiol. **11**:598-601.