DEOXYRIBONUCLEIC ACID-ENVELOPE COMPLEXES
FROM ESCHERICHIA COLI

A Complex-Specific Protein and Its Possible Function
for the Stability of the Complex

HANS-G. HEIDRICH and WILLIAM L. OLSEN

From the Department Hannig and Department Hofschneider, Max Planck Institut fü R Biochemie,
Martinsried b. München, West Germany. Dr. Olsen's present address is the Department of Biology and
Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

ABSTRACT

The different Escherichia coli envelope fractions (cell wall, cytoplasmic membrane,
and DNA-envelope complex fragments) were isolated by free-flow electrophoresis
and analyzed by sodium dodecylsulfate-acrylamide gel electrophoresis. The
DNA-envelope complex fragments possess a specific protein (mol wt
80,000-90,000). Upon treatment with trypsin, this protein disappears and the
complex breaks down, thus releasing DNA, cell wall, and cytoplasmic membrane.
Disaggregation of the complex can also be achieved by high salt concentrations.
Lysozyme treatment dissolves the murein layer within the complex but does not
disaggregate the complex. From these and other results on the stability of the
DNA-envelope complex, conclusions can be drawn about the possible linkage
within the described envelope particles.

Although most models that describe the control of
bacterial replication and cell division propose the
attachment of the chromosomal DNA to the
membrane, the nature of the bonds and the
structures involved in this attachment are com-
pletely unknown. Morphological studies with the
electron microscope have shown associations of
the chromosome with the bacterial cell surface or
with the intracellular mesosomes of gram-positive
bacteria (30) but have been unable to define the
association. Membrane-DNA complexes have
been isolated from bacterial lysates by density
gradient centrifugation, a procedure which en-
riches for rapidly sedimenting complexes (RSC)1

1 Abbreviations used in this paper: FFE, free-flow
electrophoresis according to Hannig; RSC, rapid sedi-
containing DNA in association with some large
cellular membranous structure (24). Membranous-
DNA complexes have also been isolated by their
affinity for magnesium-sarkosyl crystals (10, 40).
Some information has been obtained from experi-
ments of these types about the specificity of the
attached membrane-bound DNA. For example, in
Escherichia coli and Bacillus subtilis the chromo-
sonal origin (12, 39) and the active replicating site
(15, 36) as well as many viral DNAs (35) have been
found to be associated with membrane complexes.
However, the cell lysis and membrane isolation

menting complex; sarkosyl, sodium lauroyl sarcosinate,
NL 30; SDH, succinate dehydrogenase (E.C. 1.3.99.1),
expressed in moles/minute per milligram protein; SDS,
sodium dodecylsulfate, TRA, triethanolamine.
were prepared and DNA-envelope complexes isolated by AutoAnalyzer (Technicon Corp., Ardsley, N.Y.). Lipofractions was measured on an automated Technicon previously described (27). The protein concentration of the preparative free-flow electrophoresis (FFE) (16) as previously described (27). The following enzymes were used: trypsin (EC 3.4.4.4) was Trypure-Novob from Novo Industrie (Mainz) and contained no chymotrypsin activity. Lysozyme (EC 3.2.1.17) and contained no chymotrypsin activity. Lysozyme (EC 3.2.1.17) was obtained from Serva (Heidelberg). [3H]Thymidine (29 Ci/mmol) was obtained from The Radiochemical Centre (Amersham, England).

**Buffers and Reagents**

The buffers used have been previously described (27). The following enzymes were used: trypsin (EC 3.4.4.4) was Trypure-Novob from Novo Industrie (Mainz) and contained no chymotrypsin activity. Lysozyme (EC 3.2.1.17) and contained no chymotrypsin activity. Lysozyme (EC 3.2.1.17) was obtained from Serva (Heidelberg). [3H]Thymidine (29 Ci/mmol) was obtained from The Radiochemical Centre (Amersham, England).

**Polyacrylamide Gel Electrophoresis**

Electrophoresis in acrylamide slab gels (7.5%) was performed in a vertical gel electrophoresis cell (E.C. Apparatus Corp., St. Petersburg, Flor.) as previously described (17), with the addition of 0.1% sodium dodecylsulfate (SDS) to the gel and the electrophoresis buffer. Gels were run for 150 min at 3°C. The amperage during the run was maintained at 60 mA while the voltage increased from 200 V at the beginning to 270 V at the end of the run. Samples were prepared for electrophoresis by centrifuging the cell fragments and treating the resulting pellets for 20 min at 70°C with 0.01 M phosphate buffer, pH 7.4, containing 0.02 mg SDS, 0.03 mg mercaptoethanol, and 0.1 ml of glycerol per ml of buffer. The solubilized samples were then centrifuged at 50,000 g for 15 min, and 10 μl of the supernate (40–80 μg of protein) was applied to the gel. After electrophoresis, the gels were fixed for 2 h in 50% trichloroacetic acid, stained in a 1% Coomassie brilliant blue solution (in 50% trichloroacetic acid) for 2 h, and then destained in 10% acetic acid overnight. The following day, the gels were stained by

**MATERIALS AND METHODS**

**Bacteria and Radioactive Labeling**

*E. coli* KMBL42, grown in Difco antibiotic media no. 3 (Difco Laboratories, Detroit, Mich.), was used in all experiments. The bacterial cells were grown in 3-10 liters of medium at 37°C with aeration to 2-3 × 10^8 cells/ml (OD = 0.3–0.4, Beckman colorimeter C, green filter, Beckman Instruments, Fullerton, Calif.). Before harvesting, 500–1,500 ml of the culture was removed to a separate flask and labeled with 3 μCi [3H]thymidine/ml for approximately two cell generations.

**Isolation of the DNA-Envelope Complex**

Unless otherwise described, crude envelope fractions were prepared and DNA-envelope complexes isolated by preparative free-flow electrophoresis (FFE) (16) as previously described (27). The protein concentration of the fractions was measured on an automated Technicon AutoAnalyzer (Technicon Corp., Ardsley, N.Y.). Lipo-

HEIDRICH AND OLSEN *DNA-Envelope Complexes for E. coli* 445
passage through a staining series (11). The gel was held at each staining step for 2 h. Finally, the gels were again destained in 10% acetic acid overnight.

RESULTS

Isolation of the DNA-Envelope Complex

The DNA-envelope complex was isolated from enriched *E. coli* cell wall preparations (27) by FFE (16). Fig. 1 A shows the electrophoretic separation of the [H]DNA-envelope complex (fractions 17–20) from the cell wall fragments (fractions 21–26). The isolated membrane fragments (Fig. 1 B, fractions 29–36) which, due to a lower surface charge, migrate less strongly toward the anode do not contain [H]-labeled DNA. The peak cell wall fractions have a lower specific SDH activity (0.8 × 10⁻⁸ mol/min per mg protein) than the membrane fractions (9 × 10⁻⁸), while the complex fractions have intermediate values (2 × 10⁻⁸). Free DNA migrates slightly farther toward the anode than does the DNA-envelope complex fragments (Fig. 1 C). That the [H]DNA in fractions 17–20 is attached to cellular fragments is also seen by sedimentation of the material through sucrose gradients and its recovery as a RSC at the top of the 65% sucrose shelf (see Fig. 12).

Electron microscope observation of the DNA-envelope complex Fig. 2 shows an electron micrograph of a thin section prepared from isolated DNA-envelope complexes. The fragments contain sections of both the cell wall and inner membrane which, at zones within the complex, adhere together. These sharply defined regions, the wall/membrane adhesion zones, make up only part of the complex. The adhesion zones vary in length between 150 and 250 nm as measured from sections of about 100 particles. Therefore, it is almost impossible in the present stage of this study to speculate about their actual size, shape, or number per total surface area of the *E. coli* cell (20). For the same reason, it appears unrealistic to compare them with Bayer's contact zones (2) in plasmolyzed cells as has been proposed earlier (27).

Samples of the isolated DNA-envelope complex were also observed in the electron microscope after preparation by the Kleinschmidt procedure (25). Fig. 3 shows several representative complex fragments. Most of the envelope particles contain an attached piece of DNA. Complexes having two to six (or even more) strands of attached DNA are also seen. In most of the complex fragments, the DNA appears to originate from within the center of the curled structure.

Buoyant Densities of the *E. Coli*

Envelope Components

The DNA-envelope complex, which contains both wall and membrane, is found during the isolation procedure after centrifugation in the discontinuous sucrose gradients (27) in the wall-enriched fractions. The determination of the actual buoyant density (ρ) of the complex relative to the wall and membrane is shown in Fig. 4. In these gradients, the envelope fragments sediment to positions corresponding to the following buoyant densities: wall ρ = 1.205, complex ρ = 1.195, membrane ρ = 1.175. There is also a small shoulder of membrane fragments which have a lighter density. Similar results for the density distributions of the envelope components have been reported for *E. coli* (19, 37) and for *Salmonella typhimurium* (28, 1)
FIGURE 2 Electron micrograph (thin section) of DNA-envelope complex fragments (fractions 16-20 from Fig. 1 A). Calibration = 0.2 μm; × 100,000, insert × 200,000.
M-Band Formation by the DNA-Envelope Complex

A technique has been described for the isolation of bacterial membrane-DNA complexes based on the ability of the membrane to bind hydrophobically to crystals of magnesium-sarkosyl (10, 40). After a short centrifugation in a sucrose gradient, the crystals and the membranous-DNA complexes are found as a sharp band (M band) in the middle of the gradient. Since this technique has been used to demonstrate the membrane attachment of bacterial and phage DNAs (40, 35), it was of interest to determine if the DNA-envelope complex as isolated by the FFE would bind to the magnesium-sarkosyl crystals and could subsequently be found.
in the M band. Fig. 5 shows that when a preparation of DNA-envelope fragments was mixed with magnesium-sarkosyl crystals, about one-half of the \[^{3}H\]DNA could be recovered in the M-band fractions after centrifugation. Since all the \[^{3}H\]DNA in the preparation was attached to envelope fragments which could be identified as RSCs, it was assumed that the DNA not recovered in the M band probably was attached to envelope fragments which were too small to bind to the detergent crystals.

**Polyacrylamide Gel Electrophoresis**

The DNA-envelope complex has been shown by analysis of marker enzymes, by electron microscopy, and by density equilibrium centrifugation to be composed of cell wall and inner membrane...
FIGURE 4  Equilibrium density gradient centrifugation of E. coli envelope components. Samples of a cell wall, membrane, and DNA-envelope complex isolated by FFE (Fig. 1) were layered on linear 30–55% sucrose gradients (in TRA/Mg \(^{2+}\) buffer) and centrifuged in the SW 27.1 rotor for 16 h at 25,000 rpm (85,000 g) at 4°C. Fractions were collected from the bottom of the tubes and assayed for radioactivity or absorbance at 280 \(\mu\)m. The density of the fractions was determined by refractometry. The figure is a composite of the three gradients in which the sucrose concentrations of each corresponding fractions were identical. ●, [3H]DNA-envelope complex; ○, A\(_{260}\) of wall; x, A\(_{280}\) of the membrane; □, density.

FIGURE 5  M band of the DNA-envelope complex. A sample of the DNA-envelope complex isolated by FFE was mixed with sarkosyl and MgCl\(_2\) and centrifuged in the M-band gradient as described in Materials and Methods.

components (27). Polyacrylamide gel electrophoresis analysis of the DNA envelope complex should, therefore, reveal the presence of all the E. coli cell envelope proteins plus an enrichment for any protein specific for the complex. The results of polyacrylamide gel electrophoresis analysis of E. coli envelope proteins are very dependent upon the protein solubilization procedure used (23, 29), and it is therefore difficult to compare new data with other published experiments. To observe differences in the protein content of the various fractions isolated by FFE, solubilization with 2% SDS at 70°C was found to produce good resolution and to yield the most reproducible results.

In Fig. 6 the prominent bands 5 and 6 are found in the unfractuated envelope (A) and in all the envelope subfractions (B–D). These bands appear to correspond to the principle envelope bands described by Henning et al. (18), and upon solubilization at 100°C they show a behavior similar to that shown in the work mentioned. These two bands 5 and 6 will not be discussed here.

In gel B of Fig. 6, several major proteins (bands 3, 4, 7, and 8) can be identified as being characteristic for the cell wall. These bands were present in all the gels run and were always reproducible, whereas some minor bands were not. Therefore, only bands 3, 4, 7, and 8 were considered to be reliable markers for the cell wall. The same is true for band 2 in gel C which was chosen as the reliable marker for the inner membrane, even though there were some minor proteins which might be characteristic for the cytoplasmic membrane. All the described proteins can also be seen in the unfractuated envelope A.

The polypeptides of the DNA-envelope complex (gel D, Fig. 6) correspond to all the polypeptides found in the total envelope A plus a band which is highly and reproducibly enriched in the complex fragments. This band was chosen as the marker protein for the complex even though there are some minor bands present. This main marker protein has a mol wt of 80,000–90,000. The migration of this band 1 was not altered upon solubilization of the complex in SDS medium at 100°C. This complex-specific protein may correspond to a protein identified by Schnaitman (33). He reported the presence of a high molecular weight Triton X-100-soluble protein (i.e., a membrane protein) which was found only in the envelope regions where the membrane is attached to the cell wall. A small amount of band-1 protein is also seen in the cell wall. This is probably due to migration in the FFE of some complex fragments which have extremely small DNA molecules together with the normal cell wall fragments. These are sometimes also seen in the electron microscope.

**Effect of Lysozyme on the DNA-Envelope Complex**

Since the DNA-envelope complex fragments contain all the structural layers of the cell envelope
FIGURE 6 SDS-acrylamide slab gels of the different *E. coli* envelope fractions as isolated by FFE. (A) Unfractionated envelope; (B) cell wall (fractions 23-26 from Fig. 1 A); (C) cytoplasmic membrane (fractions 32-36 from Fig. 1 B); (D) DNA-envelope complex (fractions 16-20 from Fig. 1 A); (E) DNA-envelope complex after treatment with trypsin (20 μg/mg protein for 30 min at 20°C). Note here that band 1 has disappeared. (F) DNA-envelope complex after treatment with lysozyme (30 μg/mg protein for 60 min at 20°C). Band 1 is still present. Gels were run and stained as described in Materials and Methods.

FIGURE 7 Effect of lysozyme on the electrophoretic mobility of the isolated DNA-envelope complex. A wall-enriched preparation isolated from 6 liters of [*H]*thymidine-labeled cells was electrophoresed as described in Fig. 1 A. The peak fractions of [*H]*-labeled DNA-envelope fragments were pooled and the fragments concentrated by centrifugation at 183,000 g for 30 min. The pellets were suspended in TRA/Mg⁺⁺ sucrose buffer at a concentration of 3 mg protein/ml. One-half of the DNA-envelope preparation was re-electrophoresed after an incubation period of 1 h at room temperature (A). To the other half, lysozyme was added (100 μg/ml) and the preparation was incubated for 1 h at 25°C before re-electrophoresis (B). Fractions were collected and aliquots were assayed for protein (●) and [*H] radioactivity (○).
Figure 8: Electron micrograph of DNA-envelope complex after treatment with lysozyme. The murein layer has disappeared (arrow) but the close adhesion between wall and membrane and the basic shape of the complex fragments have been kept. Calibration = 0.2 μm. × 100,000; insert, × 200,000.
zyme-treated complex fragments is also shown by its sedimentation as a RSC in sucrose gradients (see Fig. 12). It should be noted also that after the lysozyme treatment of the DNA-envelope complex, no DNA or protein was found to have migrated into the region of the electrophoresis corresponding to membrane fragments. Electron micrographs of thin sections of the lysozyme-treated complexes (Fig. 8) show that, although the middle peptidoglycan layer of the complex has been almost completely removed in most of the fragments, the complexes have retained their basic morphology. The wall and the membrane have not separated from each other, and the typical shape has been retained.

**Effect of Trypsin on the DNA-Envelope Complex**

It has been suggested that trypsin cleaves specifically the lipoprotein that links the outer membrane of the cell wall (3, 18) to the peptidoglycan layer. When isolated envelopes of *S. typhimurium* or of *E. coli* are treated with trypsin, the associations between the wall and the inner membrane are also abolished (5) and the membrane no longer adheres to the wall at the wall/membrane adhesion zones. When the DNA-envelope complex is treated with trypsin, it loses most of its DNA and releases membrane fragments (Fig. 9). The [*H]DNA present in fraction 18 (Fig. 9 B) was shown to be unattached to RSC by centrifugation in sucrose gradients (see Fig. 12). However, the type of membrane fragments separated by electrophoresis (Fig. 9 B, fractions 34–38) could not be identified by assaying SDH activity since it was shown in control experiments that trypsin completely inactivates this SDH in membrane preparations. Therefore, the fragments were characterized by observation of their morphology in the electron microscope. Fig. 10 a is an electron micrograph of the envelope fragments from the main protein peak in Fig. 9 B (i.e., fractions 24–26). In all the complex fragments, the wall/membrane adhesion zones have been destroyed. In most fragments a very diffuse membrane can be seen within the curled structure of the complex fragment but in no case is the membrane in close contact with the cell wall as is seen in the untreated complex (Fig. 2). Also, the normally heavy staining peptidoglycan layer of the wall has become diffuse after the trypsin treatment. Fig. 10 b is an electron micrograph of the material isolated in the right shoulder of the electrophoresis run shown in Fig. 9 B (fractions 34–38). The material is composed of double-layered membrane vesicles which were apparently released from the complex by the trypsin treatment.

Polyacrylamide gel electrophoresis analysis of the proteins of the DNA-envelope complex after trypsin treatment indicated clearly that the complex-specific protein in band 1 (gel D, Fig. 6) has completely disappeared (gel E, Fig. 6). The additional bands in gel E when compared with gel D are probably digestion products which stem from the trypsin treatment.

**Effect of DNase on the DNA-Envelope Complex**

In an earlier paper (27), it was reported that after DNase treatment of the complex, the DNA had been removed; but it could never be proven unequivocally whether the last very short pieces of DNA were completely detached from the envelope. Electron micrographs (not shown here) indicated that, even after DNase treatment, wall and membrane are still together. In electrophoresis

---

**Figure 9** Effect of trypsin on the electrophoretic mobility of the isolated DNA-envelope complex. DNA-envelope complex was isolated as in Fig. 7 and divided into two parts. One part was re-electrophoresed without further treatment (A), while the other (B) was treated with 1 µg trypsin/50 µg of protein. After the 20-min incubation at room temperature, the preparation was cooled in an ice bath and immediately re-electrophoresed. •, protein; O, [*H]DNA.
FIGURE 10 a  Electron micrographs of DNA-envelope complex after treatment with trypsin and after FFE. Material from fractions 24–26 of Fig. 9 B. The close adhesion between wall and membrane has disappeared arrow, and the membrane is loose and very diffuse. Some complex pieces have completely lost the membrane. Calibration = 0.2 μm; × 100,000.
Electron micrographs of DNA-envelope complex after treatment with trypsin and after FFE. Material from fractions 34-38 of which has been loosened from the complex by trypsin. Calibration = 0.2 μm; insert × 100,000; inset × 200,000.
Effect of salt concentration on the electrophoretic mobility of the DNA-envelope complex. DNA-envelope complex was isolated as in Fig. 7 and divided into three parts. One part was given no further treatment (A). NaCl was added to the other two parts to final molarities of 0.5 M (B) and 2.5 M (C). After 30-min incubation on ice, all the samples were dialyzed at 4°C against several changes of TRA/Mg++ sucrose buffer. After dialysis, the samples were re-electrophoresed. ●, protein, O, [3H]DNA.

Salt Stability

It has recently been proposed that the binding of the membrane and cell wall at the adhesion zones involves ionic interactions (6). This idea was tested by suspending the isolated DNA-envelope complex in buffer containing a high concentration of NaCl and then subjecting the samples to re-electrophoresis. Several concentrations of NaCl were tested. The results of the exposure of the complex to 0.5 M and 2.5 M NaCl and the electrophoresis runs of this material are shown in Fig. 11. Most of the DNA is separated from the complex and migrates into the position of free DNA (see Fig. 1 C). The electrophoretic migration of the fragments is also slightly altered, and some protein is found in the region corresponding to membrane fragments. Assay of the SDH activity in these fragments after exposure of the fragments to high salt showed no activities in all fractions. While the SDH protein is assumed to be firmly associated with the membrane, it may be able to be dissociated by the high ionic strength used. Therefore, to test if the loss of SDH activity was due to the exposure of the membrane to the high ionic strength buffer, purified E. coli inner membranes were suspended in 2.5 M NaCl for 30 min and after dialysis were assayed for SDH activity. Again, the SDH activity was

Sucrose gradient centrifugation of isolated DNA-envelope complexes. DNA-envelope complexes were isolated as described in Fig. 1. Samples of the complex preparation were exposed to various treatments and then layered on 20-35% sucrose gradients (in TRA/Mg++ buffer) over a 65% sucrose shelf. The gradients were centrifuged in a Beckman W SW50.1 rotor at 40,000 rpm (149,000 g) for 40 min at 4°C. Fractions of three drops each were collected from the bottom of the tube on filter paper disks, washed with cold 5% trichloracetic acid, and assayed for radioactivity as previously described (25). Sedimentation is from right to left. (A) No treatment. (B) DNA-envelope complex incubated with 100 µg/ml lysozyme for 1 h at room temperature. (C) DNA-envelope complex incubated with 1 µg trypsin/50 µg of protein for 20 min at RT. (D) Complex plus 1% SDS. ●, [3H]radioactivity.
significantly decreased. Although the biochemical assay could not demonstrate the separation of wall and membrane (like after trypsin treatment), observation of the fragments, after exposure to the high salt, in thin sections in the EM indicate significant alteration of the structure of the complex. After the exposure to high salt, the membrane appears diffuse, is pulled into the center of the complex, and is no longer in association with the wall at the adhesion zones. This material looks like the trypsin-treated complex.

The 80–90 K protein disappeared after the breakdown of the complex with trypsin. In agreement with this result, this protein could no longer be detected by gel electrophoresis analysis after dissociation of the complex with high salt.

**Stability of the DNA-Envelope Complex**

The effect of several other treatments on the stability of the DNA-envelope complex was tested. An assay for the attachment of the DNA to the envelope fragments is the centrifugation of the material through sucrose gradients and the recovery of the [H]DNA as a rapidly sedimenting complex (RSC) on a shelf of high density sucrose. During the short centrifugation period used, free DNA will remain at the top of the gradient. This assay, however, can only measure cosedimentation of the DNA with large envelope structures and can not preclude the possibility of its entrapment within closed membrane vesicles. Fig. 12 is a profile of four such sucrose gradients. Treatment of the DNA-complex with 1% SDS completely releases the DNA from the complex, while trypsin digestion under the incubation conditions used freed about 70% of the DNA from the RSCs. After lysozyme treatment, the DNA still sediments through the gradient as a RSC. The data from these and other similar experiments are summarized in Table 1. The complex is labile upon storage at 4°C but relatively stable when stored frozen at −20°C. The complex is unstable upon treatment with agents that disrupt ionic interactions (i.e., ionic detergents and high salt concentrations), while nonionic detergents do not appreciably dissociate the complex. Triton X-100 which, in the presence of Mg++, has been shown to solubilize only proteins of the inner membrane (7, 33) does not release DNA from the RSC. It appears that the 80–90 K protein is always present when the complex is intact, even after lysozyme or DNase treatment. As soon as the complex is broken down, for example by trypsin, high salt or ionic detergents, the protein cannot be found.

**DISCUSSION**

Previously, it has been shown that the *E. coli* chromosomal DNA is bound to the cell envelope at the wall/membrane adhesion zones (27). From the electron micrographs of several of the DNA-envelope complex fragments in Fig. 3, it can be seen that most of the DNA molecules appear to be attached to a single region within the envelope fragments and that often several strands appear to

### Table I

**Stability of the DNA-Envelope Complex**

| Treatment          | DNA recovered as RSC % |
|--------------------|------------------------|
| None               | 100                    |
| 1% SDS             | 0                      |
| 1% Brij 58         | 90                     |
| 1% sodium deoxycholate | 50                   |
| 1% Triton X100     | 100                    |
| 1% sarkosyl*       | 20                     |
| NaCl 0.05 M        | 100                    |
| 0.1                | 78                     |
| 0.5                | 38                     |
| 1.0                | 28                     |
| 1.5                | 29                     |
| 3.0                | 30                     |
| Pronase‡           | 0                      |
| Lysozyme§          | 100                    |
| Trypsin‖           | 30                     |
| DNase*             | 0                      |
| Storage at 4°C     | 5–45                   |
| −20°C              | 95                     |

Samples of the DNA-envelope complex were exposed to the indicated treatments and analyzed by sucrose gradient centrifugation as described in Fig. 12. The results are expressed as the percent of the total [H]DNA added to the gradient which is recovered as a RSC at the 65% sucrose shelf after centrifugation.

* The sample to be treated with sarkosyl was first dialysed against TRA/sucrose buffer to remove the Mg++. Then centrifuged in gradients prepared in buffer without Mg++.

‡ Pronase treatment was carried out with 20 µg enzyme/ml for 30 min at room temperature.

§ Lysozyme 100 µg/ml for 30 min at room temperature.

‖ Trypsin digestion was carried out with 20 µg/ml for 20 min at room temperature.

* DNase 10 µg/ml for 30 min at room temperature.
originates from the same site. That there would be more than one DNA strand attached to the same binding location is consistent with the recently proposed topology for a bi-directionally replicating circular chromosome containing several replication forks (9). It is not yet known which regions of the chromosome are represented by the DNA bound to the envelope complex. In some of the DNA-envelope complexes, however, the DNA (see Fig. 3) resembles that proposed by Dingman (9) for the membrane attachment of the origin and replication point of the bacterial chromosome.

Several experiments were performed to determine how the membranes are held together at the adhesion zones and if the integrity of the complex is necessary for the binding of DNA. It was shown that ionic interactions are involved in holding the complex together (Table I). At high salt concentration, the entire complex is unstable; both the DNA and the membrane are released from the adhesion zone. Although the membrane vesicles which then escape from the complex (to migrate as free membrane in the FFE) cannot be identified as inner membrane vesicles by SDH activity, it can be seen in the electron microscope that the adhesion zones are disrupted and that the vesicles resemble double-layered inner membrane vesicles. The observation that trypsin treatment of the complex releases membranes and all the DNA from the complex (Figs. 9 and 10) suggests that protein is also involved in the integrity of the complex and the binding of DNA. This does not necessarily imply that covalent linkages are involved. A similar conclusion also was made by Fuchs and Hanawalt (13). Cleavage of a protein by trypsin could alter the distribution of ionic charges which could then lead to complex disruption. It has been shown that by a similarly limited trypsin treatment the lipoprotein present in the cell wall is preferentially cleaved (3, 18). It has been suggested that this molecule is involved in the attachment of the peptidoglycan to the outer membrane (4, 3, 24). However, more recently Inouye (20) has proposed a role for the lipoprotein molecules in the formation of passive diffusion pores. Braun et al. (5) have also observed that when isolated cell envelopes of S. typhimurium or of E. coli are treated with a limited concentration of trypsin, the contact associations between the wall and the membrane are abolished. A direct role of the lipoprotein in the maintenance of these associations was not implied. Instead, it was suggested that any structural transition in the wall caused by the trypsin cleavage of the lipoprotein could change the interaction of the two membranes. This interaction does not, however, require the presence of an intact peptidoglycan layer. After lysozyme treatment which cleaves the peptidoglycan and destroys the rigidity of the cell wall (8), neither the DNA nor the adhesion zones are released (Figs. 7 and 8). That lysozyme would not release DNA from the complex was expected since RSCs composed of DNA and cell membrane structures have often been isolated from E. coli cells lysed with lysozyme-EDTA. However, the digestion of the peptidoglycan may have been expected to dissociate the wall from the membrane within the complex. This was not observed. Although after the lysozyme treatment the peptidoglycan layer of the complex could no longer be seen by electron microscopy, the membrane and the DNA remained firmly attached to the wall. It appears that both the binding of DNA and the adhesion of the wall and membrane within the complex involve noncovalent interactions. Costerton et al. (6) have suggested that the wall/membrane adhesion involves an ionic attraction of the membrane to the peptidoglycan. But the results of the lysozyme treatment of the DNA-envelope complex indicate that the presence of an intact peptidoglycan is not necessary for the maintenance of the adhesion zone.

The existence of a defined zone within the cell envelope at which these ionic membrane interactions occur would suggest the presence within the membrane of a unique structural organization in either the lipid or the protein components. Gel electrophoresis analysis of the DNA-envelope complex revealed the presence of a high molecular weight complex-specific protein (band 1 of gel D, Fig. 6). Several previous reports have correlated the presence of different envelope proteins with DNA replication or with cell division (21, 22, 26, 34, 41). These proteins are smaller than the protein of band 1 which does not appear to be an aggregate since solubilization at 100°C did not alter its electrophoretic mobility. There is no indication yet of the physiological in vivo role of this complex-specific protein. The only evidence for its involvement in either the adhesion of wall and membrane or (and?) the binding of the DNA to the complex is the complete disappearance of this protein after trypsin treatment of the DNA-envelope complex. Further characterization of this protein is nec-
sary to elucidate its function. The presence of an ionic linkage between the wall and the membrane possibly involving a specific protein molecule is consistent with existing models for the structure of the E. coli cell envelope (6, 32, 14, 31). However, sufficient data are not yet available to include in these models a mechanism for the binding of DNA to the cell envelope. After this paper was finished, Sueoka and Hammers (38) published data on the isolation of DNA-membrane complexes of B. subtilis which also possess unique proteins.

We would like to thank Prof. Hannig and Prof. Hopf anoschneider for their continuous interest in this work. We would also like to acknowledge the excellent technical assistance of Ms. K. Kiersch, E. Tomaschek, and M. Wensauer.

Received for publication 5 May 1975, and in revised form 23 July 1975.

REFERENCES

1. Ames, G. F., E. N. Spudich, and H. Nikaido. 1974. Protein composition of the outer membrane of Salmonella typhimurium: effect of lipopolysaccharide mutations. J. Bacteriol. 117:406–416.

2. Bayer, A. E. 1968. Areas of adhesion between wall and membrane of Escherichia coli. J. Gen. Microbiol. 53:395–404.

3. Braun, V., and K. Rehn. 1969. Chemical characterization, spatial distribution and function of a lipoprotein of the E. coli cell wall. Eur. J. Biochem. 10:426–438.

4. Braun, V., and U. Siegel. 1970. The covalent murein-lipoprotein structure of the Escherichia coli cell wall. Eur. J. Biochem. 13:336–346.

5. Braun, V., K. Rehn, and H. Wolff. 1970. Supramolecular structure of rigid layer of the cell wall of Salmonella, Serratia, Proteus, Pseudomonas fluorescens. Number of lipoprotein molecules in a membrane layer. Biochemistry. 9:5041–5049.

6. Costerton, J. W., J. Ingram, and K. Cheng. 1974. Structure and function of cell envelope of gram-negative bacteria. Bacteriol. Rev. 38:87–110.

7. de Pampphilis, M. L., and J. Adler. 1971. Attachment of flagellar basal bodies to the cell envelope: specific attachment to the outer lipopolysaccharide membrane and the cytoplasmic membrane. J. Bacteriol. 105:396–407.

8. de Petris, A. 1967. Ultrastructure of the cell wall of Escherichia coli and chemical nature of its constituent layers. J. Ultrastruct. Res. 19:45–83.

9. Dingman, C. W. 1974. Bidirectional chromosome replication: some topological considerations. J. Theor. Biol. 43:187–195.

10. Earhart, C. F., G. Y. Tremblay, M. J. Daniels, and M. Schaechter. 1968. DNA replication studied by a new method for the isolation of cell membrane-DNA complexes. Cold Spring Harbor Symp. Quant. Biol. 33:707–710.

11. Fairbanks, G., T. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of major polypeptides of the human erythrocyte membrane. Biochemistry. 10:2606–2617.

12. Fielding, P., and C. F. Fox. 1970. Evidence for stable attachment of DNA membrane at the replication origin of Escherichia coli. Biochim. Biophys. Res. Comm. 41:157–162.

13. Fuchs, E., and P. Hanawalt. 1970. Isolation and characterization of the DNA replication complex from Escherichia coli. J. Mol. Biol. 52:301–322.

14. Glauer, A., and M. Thornley. 1969. The topography of the bacterial cell wall. Ann. Rev. Microbiol. 23:159–194.

15. Hanawalt, P., and D. Ray. 1964. Isolation of the growing point in the bacterial chromosome. Proc. Natl. Acad. Sci. U.S.A. 52:125–132.

16. Hannig, K., and H.-G. Heidrich. 1974. The use of continuous preparative free-flow electrophoresis for dissociating cell fractions and isolation of membrane components. Methods Enzymol. 31:746–761.

17. Heidrich, H.-G. 1968. New aspects on the heterogeneity of beef liver catalase. Z. Physiol. Chem. 349:873–880.

18. Henning, U., B. Hoehn, and I. Sonntag. 1973. Cell envelope and shape of Escherichia coli. Eur. J. Biochem. 39:27–36.

19. Holland, I. B., and V. Darby. 1973. Detection of a specific DNA-cyttoplasmic membrane complex in Escherichia coli by equilibrium density centrifugation on sucrose gradients. FEBS Letters 33:106–108.

20. Inouye, M. 1974. A three-dimensional molecular assembly model of a lipoprotein from the Escherichia coli outer membrane. Proc. Natl. Acad. Sci. U.S.A. 71:2396–2400.

21. Inouye, M., and J. P. Grier. 1969. A mutation which changes a membrane protein of Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 64:957–960.

22. Inouye, M., and A. P. Pardee. 1970. Changes of membrane proteins and their relation to deoxyribonucleic acid synthesis and cell division of Escherichia coli. J. Biol. Chem. 245:5813–5819.

23. Inouye, M., and M. Yee. 1973. Homogeneity of envelope proteins of Escherichia coli separated by gel electrophoresis in sodium dodecyl sulfate. J. Bacteriol. 113:304–312.

24. Klein, A., and F. Bonhoeffer. 1972. DNA replication. Ann. Rev. Biochem. 41:301–332.

25. Kleinschmidt, A. K. 1968. Monolayer techniques in electron microscopy of nucleic acid molecules. Methods Enzymol. 12:361–379.

26. Ladzinski, A., and B. Shapiro. 1973. The signifi-
cance of membrane alterations seen in DNA synthesis mutants of Escherichia coli. Biochim. Biophys. Acta 298:59-68.

27. Olsen, W. L., H.-G. Heidrich, K. Hannig, and P. H. Hofschneider. 1974. Deoxyribonucleic acid-envelope complexes isolated from Escherichia coli by free-flow electrophoresis: biochemical and electron microscope characterization. J. Bacteriol. 118:646-653.

28. Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson. 1972. Mechanism of assembly of the outer membrane of Salmonella typhimurium. J. Biol. Chem. 247:3962-3972.

29. Rice, T. W., and A. Grula. 1974. Effects of temperature on extraction and electrophoretic migration of envelope proteins from Erwinia species. Biochim. Biophys. Acta. 342:125-132.

30. Ryter, A. 1968. Association of the nucleus and the membrane of bacteria: a morphological study. Bacteriol. Rev. 32:39-54.

31. Salton, M. R. J. 1971. The bacterial membrane. In Biomembranes. E. Manson, editor. Plenum Press, Inc., New York. I:1-65.

32. Schneitman, C. A. 1971. Effect of ethylenediaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolated cell walls of Escherichia coli. J. Bacteriol. 108:553-563.

33. Schneitman, C. A. 1971. Solubilisation of the cytoplasmic membrane of Escherichia coli by Triton X-100. J. Bacteriol. 108:545-552.

34. Shapiro, B., A. Siccardi, Y. Hirota, and F. Jacob. 1970. On the process of cellular division in Escherichia coli. J. Mol. Biol. 52:75-89.

35. Siegel, P., and M. Schaechter. 1973. The role of the host cell membrane in the replication and morphogenesis of bacteriophages. Ann. Rev. Microbiol. 27:261-282.

36. Smith, D., and P. Hanawalt. 1967. Properties of the growing point region in the bacterial chromosome. Biochim. Biophys. Acta. 149:519-531.

37. Spencer, M., and J. R. Guest. 1974. Proteins of the inner membrane of Escherichia coli: identification of succinate dehydrogenase by polyacrylamide gel electrophoresis with sdh amber mutants. J. Bacteriol. 117:947-953.

38. Sueoka, N., and J. M. Hammers. 1974. Isolation of DNA-membrane complex in Bacillus subtilis. Proc. Natl. Acad. Sci. U.S.A. 71:4778-4791.

39. Sueoka, N., and W. Quinn. 1968. Membrane attachment of the chromosome replication origin in Bacillus subtilis. 33:695-705.

40. Tremblay, G. Y., M. J. Daniels, and M. Schaechter. 1969. Isolation of a cell membrane-DNA-nascent RNA complex from bacteria. J. Mol. Biol. 40:65-76.

41. Worell, A., E. Burgi, J. Robinton, and C. Carlson. 1973. Studies on the folded chromosome of Escherichia coli. Cold Spring Harbor Symp. Quant. Biol. 38:43-51.