*Proc. Nat. Acad. Sci. USA*
Vol. 71, No. 12, pp. 4787–4791, December 1974

# Isolation of DNA-Membrane Complex in *Bacillus subtilis*

**(replication)**

NOBORU SUEOKA AND JOAN M. HAMMERS

Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder Colo. 80302

*Communicated by David M. Prescott, September 13, 1974*

**ABSTRACT** **A DNA-membrane complex of *Bacillus subtilis* was separated from the bulk of the membrane by CsCl-sucrose double gradient centrifugation. The complex has a CsCl-sucrose buoyant density of 1.35–1.45 g/cm³. Gel electrophoretic analyses show that the complex has about 5% of the whole membrane proteins and contains proteins unique to the complex as well as a small amount of membrane proteins. DNA in the complex is enriched for a genetic marker close to the origin (*purA16*) and for one near the terminus (*metB5*). Artifactual formation of the complex during the lysate preparation was shown to be unlikely.**

Possible attachment of the bacterial chromosome to the cell membrane was proposed by Jacob, Brenner, and Cuzin (1), and experimental support was presented by the electron microscopic observations of association of nuclear bodies to the membrane (2). Evidence for membrane binding of the replication points has been reported as a transient enrichment of pulse-labeled [³H]thymidine in the DNA in the membrane fraction (3, 4). Subsequently, we discovered in *Bacillus subtilis* that the membrane fraction obtained from exponentially growing cells is enriched for the genetic markers close to the replication origin and the terminus when compared with internal markers, and that a pulse of [³H]thymidine at the replication origin remained in the membrane fraction after a chase by nonradioactive thymidine (5). The enrichment of the origin and terminal markers was further substantiated by examining more markers and by varying the generation time of the cell culture (6). More recently, we reported evidence that all four origins of a chromosome during synchronous dichotomous replication are associated with the membrane, where two successive initiations results in four copies of replication origins and one copy of the terminus per chromosome (7). These results support the idea that the replication origin and the terminus are permanently associated with the membrane fraction (5). This conclusion has been supported by evidence from other laboratories (8–11). Further study on the chemical nature of the association will obviously be promoted if appropriate techniques are devised to isolate the complex away from the major part of the membrane. Some success in this direction has been reported (12, 13).

## MATERIALS AND METHODS

*Strains. B. subtilis* 168TT (*thy-trp*) was used for the major part of the study, and 168 *leu8-metB5-purA16* was used as the recipient for transformation.

*Culture Media. C medium* is minimal salts plus glucose medium (14). When labeling was done with $^{35}S$, *C medium* contained the following weight-for-weight substitutions. Magnesium chloride was substituted for magnesium sulfate and ammonium chloride was used in place of ammonium sulfate. *GM-11 medium* (15) was routinely supplemented with 50 μg/ml of tryptophan and 2 μg/ml of thymine (both from Calbiochem). When labeling was with [¹⁴C]leucine, *GM-11* contained no unlabeled leucine. *Penassay medium* is Antibiotic Medium 3 (Difco). *Bott's & Wilson's Transformation Medium* (16) was supplemented with adenine, histidine, leucine, methionine, thymine, and tryptophan (50 μg/ml of each). Transformation selection plates were made with 2% agar, *C medium*, 100 μg/ml of required amino acids, and 50 μg/ml of required base.

*Preparation of Sheared Lysate. B. subtilis* 168TT was grown overnight in 5.0 ml of 1.2% Penassay supplemented with tryptophan (50 μg/ml) and thymine (2 μg/ml). A 1:20 dilution of the overnight culture was made into *GM-11*, and the cells were grown to Klett-Summerson Colorimeter unit 40 in the presence of radioactive isotopes. The cells were harvested by centrifugation in the cold (4000 rpm for 20 min). The thoroughly drained pellet was then frozen in a bath of dry ice–ethanol and stored in a freezer. The following is the lysis procedure for a cell pellet made from a 5- to 10-ml culture. The pellet is first thawed at room temperature and resuspended in 0.3 ml of Tris (50 mM)—EDTA (0.1 M)—sucrose (0.5 M or about 16% w/w), pH 8.0, 0.1 ml of lysozyme (egg white lysozyme, Worthington Co.) (2 mg/ml in 20 mM Tris, pH 8.0), and 0.04 ml of KCN (0.1 M). This was incubated at 30° for 30 min; frequent checks were made to disperse any clumping that might have occurred. The protoplasts were then burst by rapid addition of 1.06 ml of a diluting buffer, Tris (50 mM)–EDTA (0.1 M)–sucrose (0.15 M or about 5% w/w), pH 8.0. Upon successful lysis, the lysate becomes crystal clear and highly viscous. The lysate in a screw-cap vial (inner diameter 13 mm) was kept at room temperature for 5 min and then mixed on a Vortex mixer for 1 min at the highest speed setting.

*CsCl–Sucrose Double Gradient and Sucrose Step Centrifugations.* A double linear gradient of CsCl (0.5 molal to 5 molal) and sucrose (10–20% w/w) was formed in the centrifuge tube prior to the centrifugation by the peristaltic pump method (17). The initial solution was prepared at room temperature by addition of 0.5 mmol of CsCl (0.084 g) per ml of 10% sucrose (w/w) in NNET (50 mM NaCl, 20 mM EDTA, 10 mM Tris, pH 8.0), and the final solution by addition of 5 mmol of CsCl (0.84 g) per ml of 20% sucrose (w/w) in NNET. The sample was layered on top of the gradient and centrifuged in either a SW 39 rotor at 35,000 rpm or in a SW 27 rotor at 25,000 rpm for 30 min at 4°. Sucrose step centrifugation was performed by making a bottom layer of 62% sucrose in $^1/_{10}$ NET and a top layer of 20% (w/w) sucrose in $^1/_{10}$ NET (4) with a volume ratio of 1 to 4 (NET: 0.1 M

Abbreviations: M fraction, membrane fraction; F fraction, free fraction.

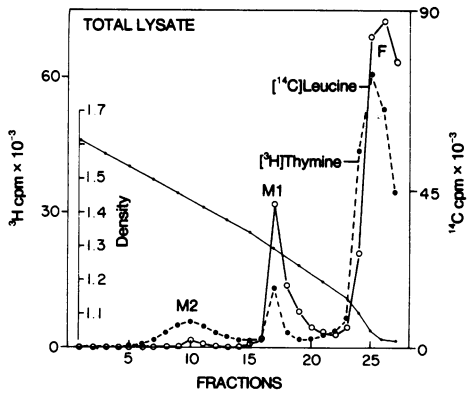


FIG. 1. CsCl–sucrose double gradient centrifugation of a sheared cell lysate ([$^3$H]DNA, [$^{14}$C]protein). *B. subtilis* 168TT was labeled by growth in the presence of [$^3$H]thymine (10 $\mu$Ci/ml, specific activity 49.5 Ci/mmol), thymine (2 $\mu$g/ml), and [$^{14}$C]-leucine (1 $\mu$Ci/ml, specific activity 304 mCi/mmol), and a sheared lysate was prepared as described in *Materials and Methods*. A 2-ml portion of the lysate was layered on top of a 15-ml CsCl–sucrose double gradient and the tube was centrifuged for 30 min in a 17-ml SW 27 rotor (4°, 25,000 rpm Spinco model L2 ultracentrifuge). Twenty-seven fractions (0.6 ml per fraction) were collected from the bottom of the tube. Aliquots of 0.1 ml were analyzed for radioactivity and one drop from each fraction was used to measure refractive index ($\eta_D^{20}$) with an Abbey refractometer. The remainder of each sample was frozen. The density of each fraction was calculated from the refractive index as follows. The densities at 20° ($\rho^{20}$) of initial and final CsCl–sucrose solutions used for making the double gradient were determined by weighing, and the refractive indices ($\eta_D^{20}$) were measured. From these measurements the following empirical equation was derived: $\rho^{20} = 9.2143\ \eta_D^{20} - 11.3868$. Likewise, the concentrations of CsCl and sucrose of each fraction can be calculated from $\rho^{20}$ by: Concentration of CsCl (molal) = $8.720\ \rho^{20} - 9.198$; concentration of sucrose (%w/w) = $19.38\ \rho^{20} - 11.55$. The above three equations are applicable only to the specified conditions of the CsCl–sucrose double gradient (see *Materials and Methods*). ○, [$^{14}$C]leucine; ●, [$^3$H]thymine; •, $\rho^{20}$.

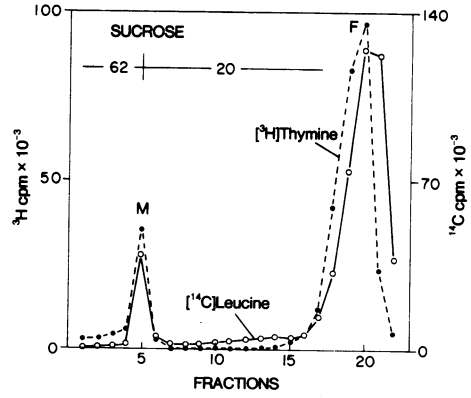


FIG. 2. Sucrose step centrifugation of a sheared cell lysate ([$^3$H]DNA, [$^{14}$C]protein). The labeled sheared lysate was prepared as in the legend of Fig. 1, except that before the lysate was mixed on a Vortex mixer it was incubated with the nonionic detergent Brij 58 (1% final concentration, 5 min, room temperature). The total sheared lysate (4 ml) from a 25-ml culture was layered on top of a step sucrose solution (10 ml of 62% sucrose in $^1/_{10}$ NET, 25 ml of 20% sucrose in $^1/_{10}$ NET) and was centrifuged in a Beckman SW 27 rotor at 25,000 rpm at 4° for 1 hr. Fractions of 1.8 ml (22 total) were collected from the bottom. NET (1.0 ml) was added to each fraction, and a 0.1-ml aliquot was used for radioactive profile analysis. The rest was kept frozen for other experiments. M, membrane fraction; F, free fraction. ●, [$^3$H]thymine; ○, [$^{14}$C]leucine.

NaCl, 10 mM EDTA, 10 mM Tris, pH 8.0.) The sample was layered on top and centrifuged at 25,000 rpm in SW 27 rotor at 4° for 60 min.

## RESULTS

*Total Lysates.* When the sheared lysate of *B. subtilis*, labeled with [$^{14}$C]leucine and [$^3$H]thymine, is centrifuged in the CsCl–sucrose double gradient at 25,000 rpm in a SW 27 rotor, two peaks, M1 and M2, are sedimented to equilibrium within 30 min (Fig. 1). On the other hand, both DNA and protein in the free fraction (F) have not yet sedimented far. The M1 peak is rather sharp with peak density of 1.28 g/cm$^3$, while the M2 peak is broad with a density range of 1.35–1.45 g/cm$^3$. Assuming that the [$^{14}$C]leucine and [$^3$H]thymine contents represent relative measures of proteins and DNA in both peaks, M2 has about 40 times more DNA than M1 per equivalent amount of protein. Both M1 and M2 are practically in equilibrium within 15 min of the start of centrifugation, since their positions hardly change by further centrifugation.

*Membrane Fraction.* When the total sheared lysate is centrifuged in a 20–62% sucrose step solution, the fraction at the interphase (Fig. 2) contains membrane material and is conventionally called the membrane (M) fraction, which contains 5–20% of the total DNA and protein, depending on the preparation. Further centrifugation of the M fraction in a CsCl–sucrose double gradient gives two peaks of protein (M1 and M2) which correspond to 20–30% and 1–2% of the total protein, respectively. The ratio of protein in M2 to protein in M1 is therefore about 1:20. On the other hand, an appreciable amount of DNA is liberated from the M fraction by the CsCl–sucrose centrifugation and remains at the top of the double gradient. The amount of the freed DNA varies considerably (10–50% of the M fraction DNA) and is roughly proportional to the amount of M fraction DNA in the preceding sucrose step centrifugation. This indicates that more weakly bound (possibly nonspecific) DNA may be removed by the high salt centrifugation. The DNA amount of the M1 peak, however, seems more variable from sample to sample than that of the M2 peak. Contents of RNA in the three fractions were examined by labeling the cells with [$^{14}$C]uracil and [$^3$H]thymine. The results indicate that the RNA content is not substantial in the fractions M1 and M2, eliminating, e.g., the possibility that ribosomes are a major component of these fractions.

*Protein Patterns of Fractions F, M1, and M2.* After the cells were labeled with $^{35}$S, the proteins in three fractions, F, M1, and M2, were analyzed by polyacrylamide gel electrophoresis. The results indicate that while the M1 fraction has a profile similar to the M fraction, the M2 fraction has a distinctly different and simpler profile (Fig. 3). Besides the four major proteins in M2, a faint background of the protein pattern—similar to M or M1—is visible. This supports the notion that the M2 fraction carries a small amount of membrane material in addition to the major proteins unique to the complex.

*Tests for Artifactual Nature of M1 and M2.* In order to test whether or not DNA–protein complexes in M1 and M2 are a result of artifactual aggregation during cell lysate preparation, the following experiments were performed. The first experiment is to add exogenous native or denatured forms of puri-

TABLE 1. *Binding of exogenous DNA to membrane fractions*

| Preparation | DNA | M2 (%) | M1 (%) | F (%) | Total cpm |
|---|---|---|---|---|---|
| | *A. Exogenous:* native[$^3$H]DNA | | | | |
| Cells | Endo. ($^{14}$C) | 7.9 | 3.0 | 89.1 | 63,480 |
| | Exo. ($^3$H) | 0.7 | 0.9 | 98.4 | 53,304 |
| Protoplasts | Endo. ($^{14}$C) | 4.1 | 3.0 | 92.9 | 52,860 |
| | Exo. ($^3$H) | 0.2 | 1.1 | 98.8 | 42,131 |
| Sheared lysate | Endo. ($^{14}$C) | 3.4 | 2.6 | 94.0 | 52,296 |
| | Exp. ($^3$H) | 0.2 | 0.5 | 99.3 | 51,060 |
| | *B. Exogenous:* denatured [$^3$H]DNA | | | | |
| Protoplasts | Endo. ($^{14}$C) | 11.0 | 7.8 | 81.2 | 93,723 |
| | Exo. ($^3$H) | 0.6 | 1.1 | 98.3 | 65,119 |
| Sheared lysate | Endo. ($^{14}$C) | 13.9 | 14.7 | 71.4 | 90,923 |
| | Exo. ($^3$H) | 1.7 | 0.9 | 97.4 | 66,103 |

Cells of strain 168TT were uniformly labeled in 10 ml of *GM-11* medium with [$^{14}$C]thymine (1 $\mu$Ci/ml, 57.3 mCi/mmol). The cells were harvested at 40 Klett units, suspended in the lysing solution, and divided into three aliquots (0.11 ml each). *B. subtilis* DNA (43,000 cpm of $^3$H/$\mu$g) was prepared by labeling strain 168TT with [$^3$H]thymine. In experiment A, 2.3 $\mu$g of [$^3$H]DNA (in 0.01 ml) was added to the cell suspension in the lysing solution, to protoplasts, and to sheared lysate. The amount of exogenous DNA is about half of the endogenous DNA. The sheared lysates of these three preparations were analyzed in the CsCl–sucrose double gradient. In experiment B, 0.1 ml of the [$^3$H]DNA solution (230 $\mu$g/ml) was diluted 10-fold in 15 mM NaCl–1.5 mM $Na_3$ citrate, heated at 94° for 10 min, and quickly cooled in ice water. Aliquots (0.1 ml) of this denatured [$^3$H]DNA were added to the cell suspension in the lysing solution, to protoplasts, and to the sheared lysates as described for experiment A. The first sample (cells) did not lead to lysis, and thus could not be analyzed. The sheared lysates of the other two samples were analyzed by the double gradient centrifugation.

fied *B. subtilis* DNA ($^3$H-labeled), at the protoplast and lysate stages, to the cells whose DNA has been uniformly labeled with [$^{14}$C]thymine. Usually very small amounts of exogenous DNA are trapped in the M1 and M2 fractions (Table 1), indicating that artifactual trapping of exogenous DNA in membrane fractions is small, if any. The second set of experiments is designed to test whether or not the endogenous DNA ($^3$H-labeled) can be diluted out by addition of excess cold DNA from outside during lysate preparation. To four aliquots of protoplasts of 168TT cells from a 25 ml-culture in *GM-11* labeled with [$^3$H]thymine (1 $\mu$Ci/ml, 49.5 Ci/mmol), various amounts (0, 10, 50, and 100 $\mu$g) of nonradioactive *B. subtilis* DNA were added from outside. In this experiment, the amount of endogenous DNA in each sample was about 10 $\mu$g. There were no signs of decrease in the amount of [$^3$H]DNA (endogenous) in the fractions M1 and M2 by the addition of exogenous nonradioactive DNA. These results favor the notion that the DNA–protein complex in the M2 fraction has been formed before the cells are opened.

*Transforming Activities of F, M1, and M2.* Fig. 4 shows that in a CsCl–sucrose gradient transforming activity coincides approximately with the [$^{14}$C]thymine profiles of M1 and M2 and confirms that the radioactivity represents DNA. The marker *purA16* is located near the origin (18), *leu8* in the middle, and *metB5* close to the terminus (19) of the *B. subtilis* chromosome. In the free fraction (F), however, the specific transforming activity relative to $^{14}$C is much lower than that of the M fractions, and transforming activity and $^{14}$C do not

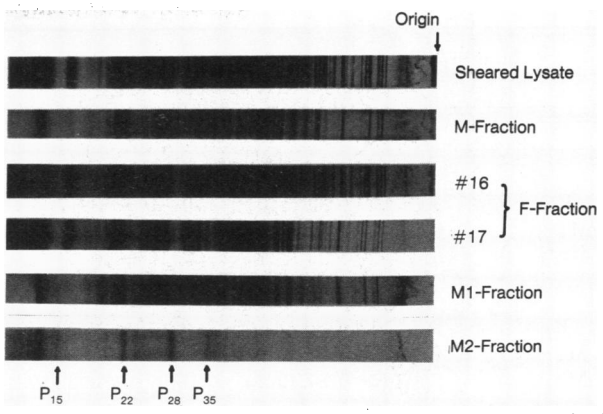


FIG. 3. Sodium dodecyl sulfate–polyacrylamide gel analysis of proteins of F, M1, and M2 fractions. *Preparation of M, F, M1, and M2 fractions.* Strain 168TT was grown in 10 ml of S-free *GM-11* with 0.1 mCi/ml of $H_2{}^{35}SO_4$ (carrier-free), 10 $\mu$g/ml of L-leucine, and 10 $\mu$Ci/ml of [$^3$H]thymine (49.5 Ci/mmol). The sheared lysate was prepared, centrifuged in a sucrose-step solution (6 ml of 62% sucrose in $^1/_{10}$ NET, 20 ml of 20% sucrose in $^1/_{10}$ NET, 25,000 rpm at 10° for 1 hr in a Beckman SW 25.1 rotor), and fractionated as described in the legend to Fig. 2. The content of each tube was diluted with 1 ml of NET; 0.1-ml aliquots were analyzed for radioactivity. One-half of the M fraction from the above sucrose centrifugation (tubes 4 and 5 combined) was diluted 3-fold with NNET and centrifuged in two CsCl–sucrose double gradients (26 ml of the gradient and 5 ml of sample in each) in an SW 25.1 rotor at 25,000 rpm at 4° for 30 min. A 0.1-ml aliquot from each fraction was assayed for radioactivity and the rest was kept in a freezer for the gel analysis. *Polyacrylamide gel electrophoresis.* M fraction and F fraction (tubes 16 and 17) from the sucrose step centrifugation, and the M2 fraction (tube 7) and the M1 fraction (tube 12) from the CsCl–sucrose double gradient centrifugation were separately dialyzed against 400 volumes of $H_2O$ overnight at 4° and lyophilized. The dry material from each fraction was dissolved in 2 ml of $H_2O$, dialyzed against $H_2O$ overnight, and lyophilized. Gel electrophoresis in 10% acrylamide in the presence of 0.1% sodium dodecyl sulfate was done according to Laemmli (29) in a slab gel apparatus (30). An autoradiogram was made on Kodak NS-2T x-ray film (30). Major proteins of the M2 fraction are indicated by P. The subscripts refer to molecular weight ($\times 10^{-3}$) of the bands.

exactly coincide. This has been previously observed in experiments with sucrose step centrifugation (5, 10, 11, 20). The reason has not been clear until now. When we treat the F fractions with phenol, however, the specific transforming activity greatly increases and becomes coincidental with the radioactive profile (Fig. 4A and B). Removal of lysozyme from the F fractions is the most likely reason for this activation, since the enzyme can certainly damage recipient cells. It is, however, important to note that transformation ratios of markers *purA16*$^+$/*leu8*$^+$ and *metB5*$^+$/*leu8*$^+$ of F fraction DNA remain similar before and after the phenol treatment (Fig. 4C). This means that there is no appreciable selective loss in transformation of particular markers by not having phenolization and, therefore, the previous conclusion on the membrane enrichment of origin and terminal markers (5, 10, 11, 20) gains further support.

In a separate experiment, dialysis and phenolization were performed throughout the entire profile. The transformation results are shown in Table 2. The ratios *purA16*$^+$/*leu8*$^+$ and *metB5*$^+$/*leu8*$^+$ show enrichment of *purA16* and *metB5* in the membrane fractions with or without phenolization. The *leu*$^+$

TABLE 2. *Effect of phenolization on transforming efficiency*

| | Membrane enrichment of *purA16* and *metB5* markers relative to *leu8* | | Membrane enrichment relative to [$^{14}$C]DNA | | |
|---|---|---|---|---|---|
| | *purA16*$^+$/*leu8*$^+$ | *metB5*$^+$/*leu8*$^+$ | *purA16*$^+$/$^{14}$C | *leu8*$^+$/$^{14}$C | *metB5*$^+$/$^{14}$C |
| | | | Dialysis only | | |
| F | 3.37 ± 0.89 (1.0)* | 0.81 ± 0.17 (1.0) | 2.8 ± 0.7 (1.0) | 1.4 ± 0.3 (1.0) | 0.8 ± 0.2 (1.0) |
| M1 | 6.89 ± 0.52 (2.1) | 1.37 ± 0.25 (1.7) | 15.5 ± 2.5 (5.5) | 2.3 ± 0.5 (1.6) | 2.7 ± 0.4 (3.4) |
| M2 | 5.55 ± 0.35 (1.7) | 1.16 ± 0.13 (1.4) | 23.6 ± 1.9 (8.4) | 4.0 ± 0.3 (2.8) | 4.7 ± 0.4 (5.9) |
| | | | Dialysis and phenolization | | |
| F | 2.61 ± 0.26 (1.0)* | 0.64 ± 0.13 (1.0) | 2.7 ± 0.5 (1.0) | 1.1 ± 0.2 (1.0) | 0.8 ± 0.2 (1.0) |
| M1 | 6.42 ± 0.61 (2.5) | 1.10 ± 0.32 (1.7) | 6.4 ± 1.5 (2.4) | 1.0 ± 0.2 (0.9) | 0.9 ± 0.2 (1.1) |
| M2 | 7.03 ± 0.87 (2.7) | 1.37 ± 0.32 (2.1) | 7.4 ± 1.2 (2.7) | 1.1 ± 0.1 (1.0) | 1.3 ± 0.2 (1.6) |

A sheared lysate of exponentially growing cells of strain 168TT (10-ml culture) was fractionated by CsCl–sucrose double gradient centrifugation. Cells were uniformly labeled with [$^{14}$C]thymine and [$^3$H]leucine as described in the legend of Fig. 4. A 0.1-ml sample of each tube was dialyzed and brought to 0.4 ml, and a 0.1-ml aliquot was brought to 0.5 ml and phenolized as described in the legend of Fig. 4. Transformation was performed for the three markers by using a 0.1-ml aliquot of each tube from both dialyzed and dialyzed-phenolized samples. Marker ratios were calculated from each tube, and the ratios within M2, M1, or F regions were averaged and shown in the table. Standard errors were also calculated for each region. The figures in parentheses are values of the M1 and M2 fractions relative to that of the F fraction.

* Relative values to F.

specific transforming activity (*leu*$^+$/$^{14}$C) is an ideal measure for comparison of the transforming efficiency between treated and untreated DNA samples, provided that the same batch of recipient cells is used. In Table 2, transforming activities of each fraction of dialyzed and dialyzed-phenolized preparations were measured on different days with different batches of recipient cells, so that absolute values of the specific activities (*purA16*$^+$/$^{14}$C, *leu8*$^+$/$^{14}$C, and *metB5*$^+$/$^{14}$C) in the two preparations are not to be compared directly. The fact that the specific activity of F fractions increases after phenolization is evident from the result in Fig. 4. While without phenolization, M1 and M2 fractions have higher specific *leu*$^+$ transforming activity than that of F fraction, with phenolization the specific activity becomes similar in all three fractions. This means that

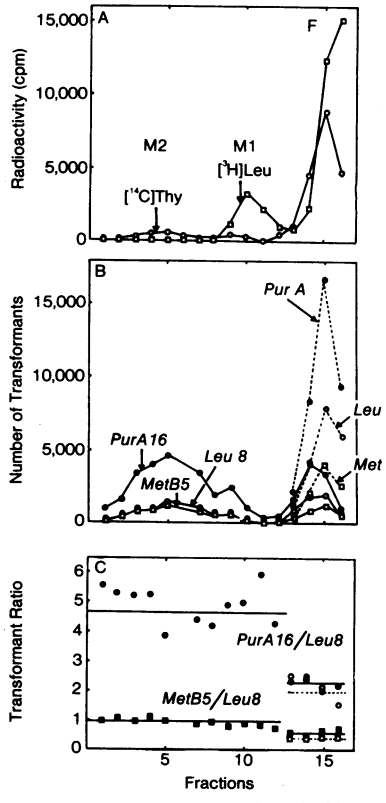


FIG. 4. Marker distribution in the CsCl–sucrose double gradient centrifugation sample of a sheared lysate. A sheared lysate of 10 ml from exponentially growing cells of strain 168TT that were uniformly labeled with [$^{14}$C]thymine (1 μCi/ml, 47.5 mCi/mmol) and [$^3$H]leucine (10 μCi/ml, 5 Ci/mmol) was centrifuged in the CsCl–sucrose double gradient (one 5-ml gradient in an SW 39 rotor) and fractionated from the bottom into 16 tubes. (A) *Radioactivity profile.* A 0.1-ml aliquot from each tube was dialyzed against 0.1 M potassium phosphate buffer (pH 7.0) overnight, with one buffer change. After dialysis, each fraction was brought to 0.4 ml by addition of the buffer. The radioactivity profiles for $^{14}$C and $^3$H were obtained by analyzing 0.1-ml aliquots of the dialyzed fractions (shown in the figure). From tubes 13–16, 0.1-ml aliquots were taken and diluted to 0.5 ml with 0.15 M NaCl–0.015 M sodium citrate and phenolized by mixing with 0.5 ml of water-saturated phenol. After centrifugation at 3,000 rpm for 10 min, the aqueous layer of each fraction was transferred to a tube, and the residual phenol was removed by shaking twice with an equal volume of ether. Radioactivity of each fraction was measured from a 0.1-ml aliquot, which gave 83, 172, 548, and 326 cpm ($^{14}$C), and 0, 4, 28, and 5 cpm ($^3$H) in tubes 13, 14, 15, and 16, respectively. (B) *Transformation profile.* Transformations for markers *purA16* (close to the origin), *leu8* (in-between), and *metB5* (close to the terminus) were performed with 0.1-ml aliquots of the dialyzed fractions (solid lines). In addition, 0.1-ml aliquots of the dialyzed-phenolized fractions of tubes 13 to 16 were used for transformation. The results (broken lines) are obtained by recalculating the number of transformants expected for the amount of DNA in the dialyzed fraction, based on the cpm ($^{14}$C) before and after phenolization, to facilitate direct comparison. (C) *Marker ratio.* Marker ratios *purA16*$^+$/*leu8*$^+$ and *metB5*$^+$/*leu8*$^+$ were calculated for each fraction from the above marker profile. Ratios calculated from the dialyzed samples are shown as *closed* circles and squares and from the dialyzed-phenolized samples as *open* circles and squares for *purA16*$^+$/*leu8*$^+$ and *metB5*$^+$/*leu8*$^+$, respectively.

DNAs in the three fractions are equally active and the apparent inefficiency of the F fraction without phenolization is due to the presence of an inhibitor of transformation (most likely lysozyme). The higher specific activities of *purA16* and *metB5* markers in M1 and M2 than in F are due to the membrane enrichment. From these results it is clear that origin and terminus markers (*purA16* and *metB5*) are relatively more frequent than the middle marker (*leu8*) in the membrane fractions (M1 and M2) compared to the free fraction (F).

## DISCUSSION

In the present work the membrane fraction (M), obtained by the step sucrose centrifugation, has been separated into two fractions by the CsCl–sucrose double gradient centrifugation. One fraction (M1) contains about 95% of the "membrane fraction" protein and the other fraction (M2) 5%, while more DNA is found in M2 than in M1. The M2 DNA–protein complex is apparently salt-resistant and seems to include a small amount of membrane material. The equilibrated M2 peak lies between CsCl concentrations of 2.8 and 3.8 molal. In this regard, DNA binding proteins of *Escherichia coli* dissociate from DNA at salt concentrations above 2 M NaCl, when soluble proteins of a cell lysate are fractionated by DNA–cellulose columns (21). Other DNA binding proteins, for example, *E. coli lac*-repressor, have little affinity for DNA above 0.2 M KCl (22). ATP-dependent DNase of *B. subtilis* is highly sensitive to salt (23). During transcription, RNA polymerase is known to be stably bound to DNA in high salt (24, 25). However, the M2 fraction does not show the clear presence of RNA polymerase core proteins, as seen in the polyacrylamide gel electrophoresis pattern (Fig. 3). The M2 proteins, therefore, seem to belong to a hitherto uncharacterized class of proteins which may bind strongly to specific sites on the chromosome.

The significance of the DNA found in the M1 fraction is not clear at the moment. However, since the membrane enrichment of *purA16* and *metB5* of M1 is similar to that of M2, it is likely that the difference between the two fractions may simply be that M1 contains a larger portion of membrane than M2, and the DNA in the M1 fraction may be the same as that of M2. Our experiments tend to eliminate the possibility that the DNA–protein complex of M2 is the result of an artifact generated during preparation of the cell lysate. The fact that the polyacrylamide gel electrophoretic pattern of M2 shows a faint background of membrane fraction supports the idea that the M2 fraction has originated from membrane and still carries some of the membrane fragments.

One of the primary objectives of this work was to isolate the chromosome–membrane attachment complex from the remaining part of the membrane. The present results indicate that the M2 fraction is most likely derived from the *in vivo* complex. In our method of isolation, the cell wall is enzymatically dissolved prior to the preparation of the lysate. This means that if there is any association of the complex to the cell wall, as has been suggested in *E. coli* (26, 27), it is lost during protoplast preparation. In the sheared lysate stage of preparation, the complex should contain the integral components of the complex. Since ionically bound proteins should subsequently fall off from the complex in high salt in the present double gradient technique, the proteins in the M2 fraction are apparently those proteins of the original complex that have strong affinity for the chromosome by interactions other than ionic binding. The M2 proteins are particularly interesting since they may bind to DNA primarily by base sequence specificity. It is clear that since the DNA in the complex includes several parts of the genome (origin, terminus, replication fork, and some unspecified sites), the M2 fraction should consist of several different complexes with potentially different proteins. The apparent simplicity of the polyacrylamide gel pattern in the M2 fraction (Fig. 3) gives encouraging thoughts about the future study of this work. During the preparation of this paper, Olsen *et al.* (28) reported isolation of the DNA–outer membrane complex from the bulk cell wall and membrane in *E. coli*.

We thank Dr. Aideen O'Sullivan and Dr. Sumi Imada for helpful discussions, Mr. E. Kauffman for initial assistance, and Dr. Masaru Imada for performing gel electrophoresis of our preparations. This work has been supported by National Science Foundation Grant GB-19560 and National Institutes of Health Grant GM-10923.

1. Jacob, F., Brenner, S. & Cuzin, F. (1963) *Cold Spring Harbor Symp. Quant. Biol.* **28**, 329–336.
2. Ryter, A. & Jacob, F. (1964) *Ann. Inst. Pasteur* **107**, 384–400.
3. Ganesan, A. T. & Lederberg, J. (1965) *Biochem. Biophys. Res. Commun.* **18**, 824–835.
4. Smith, D. W. & Hanawalt, P. C. (1967) *Biochim. Biophys. Acta* **149**, 519–531.
5. Sueoka, N. & Quinn, W. G. (1968) *Cold Spring Harbor Symp. Quant. Biol.* **33**, 695–705.
6. Sueoka, N., Bishop, R. J., Harford, N., Kennett, R. H., Quinn, W. G. & O'Sullivan, A. (1973) in *Genetics of Industrial Microorganisms*, eds., Vanet, Z., Hostatek, Z. & Cudlin, J. (Academia, Prague), pp. 73–87.
7. O'Sullivan, M. A. & Sueoka, N. (1972) *J. Mol. Biol.* **69**, 237–248.
8. Snyder, R. W. & Young, F. E. (1969) *Biochem. Biophys. Res. Commun.* **35**, 354–362.
9. Fielding, R. & Fox, C. F. (1970) *Biochem. Biophys. Res. Commun.* **41**, 157–162.
10. Ivarie, R. D. & Pene, J. J. (1970) *J. Bacteriol.* **104**, 839–850.
11. Yamaguchi, K. S., Murakami, S. & Yoshikawa, H. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1559–1565.
12. Daniels, M. J. (1971) *Biochem. J.* **122**, 197–207.
13. Ballesta, J. P., Cundliff, E., Daniels, M. J., Silverstein, J. L., Susskind, M. M. & Schaechter, M. (1972) *J. Bacteriol.* **112**, 195–199.
14. Spizizen, J. (1958) *Proc. Nat. Acad. Sci. USA* **44**, 1072–1078.
15. Kennett, R. H. & Sueoka, N. (1971) *J. Mol. Biol.* **60**, 31–44.
16. Bott, K. F. & Wilson, G. A. (1967) *J. Bacteriol.* **94**, 562–570.
17. Leif, R. C. (1968) *Anal. Biochem.* **25**, 271–282.
18. O'Sullivan, M. A. & Sueoka, N. (1967) *J. Mol. Biol.* **27**, 349–368.
19. Yoshikawa, H. & Sueoka, N. (1963) *Proc. Nat. Acad. Sci. USA* **49**, 559–566.
20. Yamaguchi, K. & Yoshikawa, H. (1973) *Nature New Biol.* **244**, 204–206.
21. Alberts, B. M., Amodio, F. J., Jenkins, M., Gutmann, E. D. & Ferris, F. L. (1968) *Cold Spring Harbor Symp. Quant. Biol.* **33**, 289–305.
22. Riggs, A. D., Suzuki, H. & Bourgeois, S. (1970) *J. Mol. Biol.* **48**, 67–83.
23. Ohi, S. & Sueoka, N. (1973) *J. Biol. Chem.* **248**, 7336–7344.
24. Anthony, D. S., Zeszoteck, E. & Goldthwait, D. A. (1966) *Proc. Nat. Acad. Sci. USA* **56**, 1026–1033.
25. Fukuda, R. & Ishihama, A. (1971) *Biochem. Biophys. Res. Commun.* **45**, 1255–1261.
26. Worcel, A. & Burgi, E. (1974) *J. Mol. Biol.* **82**, 91–105.
27. Schwarz, U., Ryter, A., Rambach, A. & Hirota, Y. (1974) *J. Mol. Bicl.*, in press.
28. Olsen, W. L., Heidrich, H. G., Hannig, K. & Hofschneider, P. H. (1973) *J. Bacteriol.* **118**, 646–653.
29. Laemmli, U. K. (1970) *Nature* **227**, 680–685.
30. Studier, F. W. (1973) *J. Mol. Biol.* **79**, 237–248.