JOURNAL OF BACTERIOLOGY, Nov. 1978, p. 799-802
0021-9193/78/0136-0799$02.00/0

Vol. 136, No. 2
Printed in U.S.A.

Chromosome-Membrane Association in *Bacillus subtilis*

IV. Further Purification of DNA-Membrane Complex by Using a Combination of Centrifugation and Electrophoresis

HIROO TOYODA, KAZUO YAMAGUCHI, AND HIROSHI YOSHIKAWA*

Cancer Research Institute, Kanazawa University, Kanazawa, Japan

Received for publication 21 August 1978

We have developed a simple procedure to purify a DNA-membrane complex from *Bacillus subtilis* by using a combination of centrifugation and electrophoresis. Several unique proteins were detected in the purified complex.

Much evidence supporting association of DNA with the cell membrane has been accumulated from studies attempting to isolate DNA-membrane complexes by various methods (2-5, 11, 14, 15, 23). DNA associates with the cell membrane at three unique sites in *Bacillus subtilis*, the replication origin (11, 17-20), the replication forks (5, 11, 20), and the replication terminus (17, 19, 20-22). Numerous attempts have been made to demonstrate a possible role of specific membrane proteins on the chromosomal replication (6, 8, 10, 15, 16). Some of them have revealed that several kinds of membrane proteins may be involved in DNA synthesis (6, 9, 10, 16).

In biochemical studies, three methods have been widely used to isolate DNA-membrane complex: (i) sucrose density gradient centrifugation to isolate fast sedimentable materials from the cell lysate (15, 19, 20); (ii) the M-band method, which is based on the affinity of membraneous materials to crystals of Mg^{2+}-Sarkosyl (2, 7); and (iii) the CsCl-sucrose double gradient method (18). However, in both i and ii, a large amount of membrane fragments was isolated together with the complex. In addition, effects of the detergent and high salt concentration on the composition of the membrane could not be neglected in the case of ii and iii. Recently, Olsen et al. (9, 14) reported a new purification method to separate the DNA-membrane complex from cell wall and bulk membrane fragments by using free-flow electrophoresis. However, they did not examine whether or not the complex contained specific sites on the *Escherichia coli* chromosome. In the present study, we isolated and purified a DNA-membrane complex containing specific sites on the *B. subtilis* chromosome by using a combination of centrifugation and electrophoresis. Several specific proteins in the complex were observed when a majority of the protein in the complex was removed by sonic oscillation.

An alkaline pH was required to eliminate a large amount of DNA fragments nonspecifically associated, probably entrapped, with the cell membrane. Therefore, the cell lysate was first prepared in an alkaline buffer (TKE_1: 20 mM tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.1; 0.1 M KCl; 1 mM ethylenediaminetetraacetic acid; and 10 mM 2-mercaptoethanol), then sheared and centrifuged in TKE_1 to get rid of DNA that was not associated with the cell membrane. Fractions containing a crude DNA-membrane complex were pooled and then dialyzed against a neutral or slightly acidic buffer such as $P_{50}E_{10}$ (50 mM sodium phosphate, pH 6.7; 10 mM ethylenediaminetetraacetic acid; and 5 mM 2-mercaptoethanol). A rapid neutralization of the crude complex is essential to stabilize the complex during electrophoresis. The crude complex was purified by electrophoresis in a sucrose density gradient (Fig. 1a). Further purification was achieved by repeated electrophoresis (Fig. 1b). Repeated electrophoresis removed more than 90% of membrane fragments which originally cosedimented with the crude DNA-membrane complex. The electrophoretic patterns of DNA and protein are not identical in the fractions containing the complex, as shown in Fig. 1b. This discrepancy was due to contamination of a large amount of DNA fragments which have been cosedimented in the crude complex. Fig. 1c shows that these DNA fragments are efficiently removed by the centrifugation after the electrophoresis. It is noted that no *purA* marker, an origin marker, was released from the complex, whereas *hisA* marker, a middle marker, was released in proportion to the bulk DNA. The marker ratio between *purA* and *hisA* was 30 to 50, indicating that the purified complex was enriched for the chromosome frag-

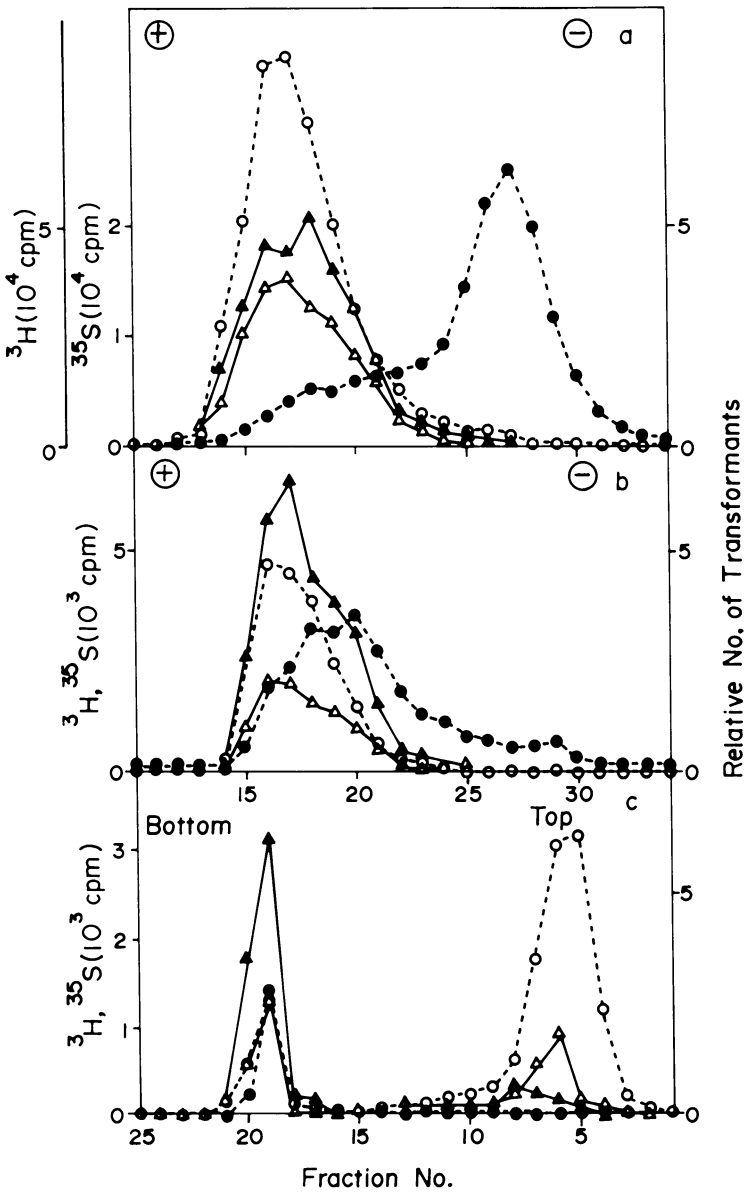

FIG. 1. *Purification of a DNA-membrane complex by a combination of electrophoresis and centrifugation. B. subtilis 168 LTT (leu trpC2 thy) was grown in enriched lowS-CG-AA supplemented with leucine (50 μg/ml), tryptophane (50 μg/ml), and 0.5% glucose, and was labeled with [^{3}H]thymidine (2.5 μCi/2.5 μg per ml) and ^{35}S (1.5 μCi/2 μg as atoms of S per ml). Cells were lysed, sheared, and centrifuged as described previously (22, 23). Fractions of a crude DNA-membrane complex were pooled and dialyzed against $P_{50}E_{10}$ buffer and subjected to electrophoresis. A zone electrophoresis in a sucrose gradient developed by Kadoya et al. (12) was used to separate the DNA-membrane complex from a bulk of membrane fragments unassociated with DNA. The sample dialyzed against $P_{50}E_{10}$ was adjusted to 5% sucrose solution containing 0.05% bromophenol blue and layered on top of a 10 to 30% linear sucrose gradient in $P_{50}E_{10}$ with a shelf of 70% sucrose in $P_{50}E_{10}$. Electrophoresis was carried out in a constant current of 20 mA at 4°C for 9.5 h. At the end of run, the sample was fractionated (20 drops each) (a). Fractions 16 through 19 were pooled and dialyzed against $P_{50}E_{10}$, then concentrated in Sephadex G-50 powder to 3 to 4 ml. The concentrated sample was divided into two. One was again subjected to electrophoresis as in a (b). The other was centrifuged in a sucrose density gradient (10 to 30% sucrose in $P_{50}E_{10}$, 64% sucrose in $P_{50}E_{10}$ as shelf) in a Beckman SW 27 rotor at 25,000 rpm for 4 h at 2°C. Thirty fractions, 1.2 ml each, were collected from the top of the gradient (c). Aliquots from all fractions were assayed for transforming activities and radioactivity as described previously (23). B. subtilis CRK3,000 (leu-8 metB5 purA16 hisA3) was used as a recipient to assay transforming activities. Symbols: ○-----○, ^3H radioactivity; ●-----●, ^{35}S radioactivity; ▲——▲, purA marker, △——△, hisA marker. (a) purA ($1 = 10^5$ transformants per ml); hisA ($1 = 5 \times 10^3$ transformants per ml). (b) purA ($1 = 10^5$ transformants per ml); hisA ($1 = 5 \times 10^3$ transformants per ml). (c) purA ($1 = 5 \times 10^3$ transformants per ml); hisA ($1 = 2 \times 10^2$ transformants per ml).*

ments near the replication origin. These values are close to the maximum enrichment value which theoretically can be obtained for the replication origin markers if the complex contained the origin specifically in addition to the replication points. Table 1 shows an increasing enrichment for the *purA* marker during the purification steps. In contrast, there was only a slight enrichment for a marker near the replication terminus, e.g., *metB*, during the purification. The complex contained phospholipid and a cell wall component as well as DNA and proteins (Table 1), suggesting that it is indeed a DNA-membrane complex.

The composition of proteins in the purified DNA-membrane complex was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins in the purified complex (fractions 16 to 17 in Fig. 1a) were compared with those of free membrane fractions (fraction 8 to 9 in Fig. 1a) in the same electrophoretic run. No significant difference was found between these preparations, indicating that major protein components in the complex were indeed derived from the cell membrane (Fig. 2a and b). At this stage, the protein content of the complex was about 3% of the total cellular protein and corresponds to 10% of the membrane protein. This amount may still be too large to identify specific proteins which are located at the binding sites. To test this possibility, the purified complex was subjected to further purification by electrophoresis after being sonically disrupted for 30 or 60 s. These treatments reduced protein contents to 0.07 and 0.03% of the total cellular protein, respectively. Five proteins with molecular weights of 38,000, 32,000, 29,000, 27,000, and 24,000 were enriched in the complex (Fig. 2c and d) after a 30-s sonic disruption. The 38,000- and 32,000-

TABLE 1. *Marker frequency analysis and the properties of a purified DNA-membrane complex*

Purification step	Marker frequency analysis (normalized)[a]		Relative amount (%) of:			
	purA/hisA	*metB/hisA*	DNA	Protein	Lipid	Wall[b]
Sheared whole lysate	3.57	0.57	100	100	100	100
SDG[c]	28.3	1.73	6.2	6.5	12.1	7.4
SDE[d]	28.3	1.73	6.2	1.4	0.4	1.0
SDG after SDE	29.6	2.10	1.5	1.4		

[a] These values were normalized by the marker frequency in *B. subtilis* 168 LTT spore DNA. The ratios of *purA/hisA* and *metB/hisA* were 1.03 and 0.57, respectively.

[b] The relative amount of wall was determined by the incorporation of [^{3}H]glucosamine (2 μCi/200 μg per ml) to the wall fraction.

[c] SDG, Sucrose density gradient centrifugation to prepare a crude DNA-membrane complex.

[d] SDE, Sucrose density gradient electrophoresis to purify a crude DNA-membrane complex.

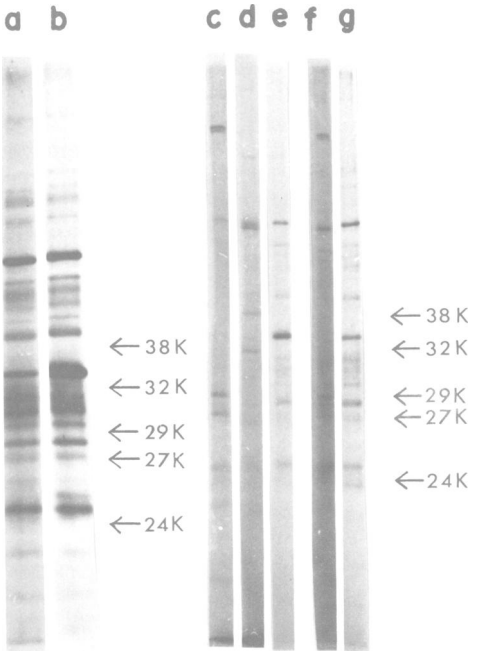

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gel analysis of proteins in the purified complex with or without sonic oscillation. The crude DNA-membrane complex labeled with ^{35}S (40 μCi/2 μg as S atom per ml) was prepared, and aliquots were sonically disrupted for 30 and 60 s by a Heat System Ultrasonicator. Samples were subjected to electrophoresis in the sucrose density gradient as in Fig. 1a. Radioactive samples in various fractions were precipitated in cold 10% trichloroacetic acid containing 1 drop of calf thymus DNA (2 mg/ml) as a carrier. Resulting precipitates were washed with acetone to remove residual trichloroacetic acid, dissolved in a small volume (40 to 50 μl) of buffer {62.5 mM tris(hydroxymethyl)aminomethane-hydrochloride [pH 6.8], 1% sodium dodecyl sulfate, and 1% 2-mercaptoethanol}, boiled for 2 min, and applied to the 10% slab gel. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli and Favre (13). The electrophoresis was carried out at a constant current of 20 mA at 20°C for 4 to 5 h. Gels were stained with 0.1% Coomasie brilliant blue in 50% trichloroacetic acid for 20 min and destained with 7.5% acetic acid. After drying under vacuum, the gel was analyzed by fluorography (1). (a) Non-sonically disrupted complex fraction; (b) non-sonically disrupted free membrane fraction; (c and d) two different complex fractions after 30-s sonic disruption; (e) free membrane fraction after 30-s sonic disruption; (f) complex fraction after 60-s sonic disruption; (g) free membrane fraction after 60-s sonic disruption.*

molecular-weight proteins disappeared when sonically disrupted for 60 s (Fig. 2f and g). Therefore, proteins with molecular weights of 29,000, 27,000, and 24,000 are most tightly bound to DNA in the complex.

We postulate that these proteins are minor components of the membrane and represent core proteins of the DNA-membrane complex. The role of the proteins in DNA binding mechanism is not known at present. A plausible model is that the proteins carry two functional sites, one for binding DNA and the other for binding membrane proteins, thus acting as linkers between DNA and the cell membrane. This model is now testable experimentally by in vitro reconstitution by using sonically disrupted, purified DNA-membrane complex.

We are grateful to S. Murakami, N. Ogasawara, and other colleagues in our laboratory for their valuable discussions and to K. Terada and K. Ando for their help and assistance in preparing the manuscript. The work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan and by a grant-in-aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

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