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Attachment of the Chromosomal Terminus of *Bacillus subtilis* to a Fast-Sedimenting Particle

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After gently lysed protoplasts of exponential phase cells of *Bacillus subtilis* were treated with restriction endonuclease *Bam*HI, 99% of the DNA did not sediment with the plasma membrane. This DNA was fractionated on sucrose gradients into (i) a fast-sedimenting fraction highly enriched for genes from the origin and terminus (*purA* and *ilvA*), (ii) a 50 to 100S component also enriched for *purA* and *ilvA*, and (iii) the bulk of the DNA. The fast-sedimenting fraction was dissociated by Sarkosyl; this fraction contained a substantial amount of protein and is probably a membrane subparticle. The S value of the 50 to 100S component was not greatly affected by Sarkosyl treatment, but these particles were unable to penetrate an agarose gel during electrophoresis and were retained by nitrocellulose filters. The terminus DNA in the fast-sedimenting fraction and the 50 to 100S component contained a large restriction fragment (1.5×10^7 to 2.0×10^7 daltons) encoding *ilvA*, *thyB*, and *ilvD*. The bulk of the SPβ prophage and *metB*, which lie to the right and left, respectively, of the *ilvA-ilvD* cluster, were not part of the complex. *citK*, which lies to the right of SPβ, appeared to be present in the fast-sedimenting complexes. The neighboring genes *kauA* and *gltA* were not part of the fast-sedimenting complexes. The presence of terminus DNA in the fast-sedimenting components was also demonstrated by a radiochemical method.

Since Jacob et al. (11) proposed an important role for the cell surface in chromosome segregation, evidence suggesting that DNA is attached to the plasma membrane has accumulated (15, 18, 27). Association of the chromosomal origins of *Escherichia coli* and *Bacillus subtilis* to the envelope layer has been demonstrated by specific radioactive labeling of the origin region and DNA-mediated transformation (5, 9, 18). Specific restriction endonuclease fragments concerned with attachment to the membrane have been identified in both of these organisms (20, 26, 32).

DNA-membrane complexes enriched for specific markers are usually isolated from hydrodynamically sheared lysates of bacteria. In most cases the DNAs in these preparations has been enriched for origin or terminus markers by no more than threefold, although only about 10% of the total DNA is present. A notable exception to this was the isolation of a particle containing the *purA* locus of *B. subtilis* in very high purity and yield (33).

The suitability of the techniques and conditions used to prepare DNA-membrane complexes has not been given much attention. Thus, agents which have major affects on the plasma membrane, such as chelating agents and nonionic detergents, are commonly used in lysis media. The effectiveness of hydrodynamic sheer in inflicting double-strand breaks on DNA depends on the chromosome being extensively dispersed (i.e., at low ionic strengths, absence of divalent counterions). The availability of restriction endonucleases has provided a tool for dissecting bacterial nucleoids which is considerably more refined than hydrodynamic shear. Our strategy has been to render the chromosome accessible to restriction endonucleases by subjecting protoplasts to a gentle hypotonic shock in the presence of a concentration of magnesium ions which stabilizes the plasma membrane and is optimal for the enzyme.

Density transfer studies have indicated that chromosome replication in *B. subtilis* proceeds bidirectionally to a terminus which appears to be in the vicinity of *citK* and *gltA* (6). The markers surrounding the terminus from *metB* to *thyA* and possibly as far as *pyrA* (Fig. 1) are enriched in certain membrane preparations. Peak enrichment appears to occur at about *citK* (5, 9). The entire region is extremely large. The prophage of SPβ, whose DNA is 120 kilobases long, is inserted between *ilvA* and *citK* (34). Therefore, the distance between *ilvA* and *citK* is about 275 kilobases, or 6% of the chromosome.

Recently, a method by which the terminus of the *B. subtilis* chromosome can be identified at the level of restriction fragments has been devised (1, 23, 24). Thus, if sporulating bacteria are labeled for a short time with [^{3}H]thymidine and

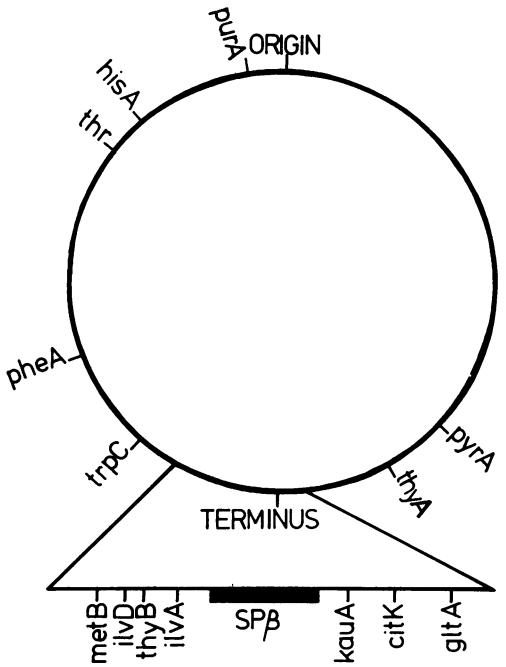

FIG. 1. Genetic map of *B. subtilis*. From Henner and Hoch (7).

then treated with an inhibitor of DNA synthesis, the only labeled spores obtained are the spores in which DNA synthesis was completed during the pulse period. The labeled DNA replicates after *metB* in germinating spores (1) and has extensive homology with phage SPβ (24). This DNA has a relatively simple restriction pattern (24), from which a map showing the exact point of termination can be constructed. We used the procedure for specific labeling to study the interaction of the terminus with the cell membrane and its subparticles.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. subtilis* strains 168 *trpC2* and 168 *trpC2 thyA thyB* were used for all labeling experiments and as sources of DNA. The following strains were used as recipients in transformation experiments: MS146 *purA16 hisA1 pheA trpC2 metB5 ilvA1 gltA292*; MS149 *purA16 hisA1 pheA trpC2 metB5 ilvA1 gltA292 thyA*; MS154 *purA16 hisA1 trpC2 metB5 ilvD15 ilvA1 thyA*; and CU1323 Δ *ilvB metB5 kauA1 citK1 gltA2* (supplied by S. A. Zahler).

Spores were germinated at 42°C with aeration in a medium containing (per liter) 10 g of casein hydrolysate, 0.2 g of L-alanine, 4 g of glucose, 7.55 g of KCl, 0.02 M $NaPO_4$ buffer (pH 7.0), 1 mM $MgSO_4$, 1 mg of $FeSO_4$, 0.1 mg of $MnSO_4$, 5 mg of tryptophan, and thymine where appropriate. Spores were heat treated at 80°C for 10 min in distilled water before they were added to the germination medium. In a typical experiment, flasks were inoculated with 10^7 spores per ml and were incubated for at least 3.5 h before harvesting. Spores were purified as described previously (28).

Low-sulfate medium. The low-sulfate medium used contained (per liter) 0.05 M KPO_4 buffer (pH 7.0), 1 mM $MgCl_2$, 1 mg of $FeCl_2$, 2 g of NH_4Cl, 0.1 mM Na_2SO_4, 4 g of glucose, 2 g of arginine, 5 mg of tryptophan, and 2.5 mg of thymine.

Radioactive labeling. Spores of *B. subtilis* 168 *trpC2 thyA thyB* containing chromosomes labeled specifically in the terminus with [^{3}H]thymidine and steady state labeled with [^{14}C]thymine were prepared by using the single-medium sporulation system described previously (24). Bacteria were grown in media containing [^{14}C]thymine (0.05 μCi/ml; specific radioactivity, 3.15 Ci/mol). At 2 h after the end of exponential growth, [^{3}H]thymidine (5 μCi/ml) was added to the culture, followed by the addition of 6-hydroxyphenylazouracil at intervals (see below).

The DNA of *thy*$^+$ strains was labeled during outgrowth of spores by adding [^{3}H]thymidine (1 μCi/ml; specific radioactivity, 52 Ci/mmol).

Steady-state labeling of DNA during germination of spores was achieved by preparing spores in the presence of [^{3}H]thymine (63 Ci/mol; 10 μg/ml) and germinating the spores in media containing [^{3}H]thymine of the same specific activity.

Protein was labeled by using $^{35}SO_4$(1 μCi/ml) in the low-sulfate medium described above.

Proteins and nucleic acids in sucrose gradients were precipitated with trichloroacetic acid (final concentration, 5%) and were collected on Whatman GF/C glass fiber filters. The filters were washed with ethanol and ether before drying and were counted in Packard scintillation fluid.

Preparation of DNA. All DNA samples used for transformation experiments were purified by using phenol. Tris-hydrochloride (pH 9.0), EDTA (disodium salt; pH 8.0), and Sarkosyl were added to DNA samples to give concentrations of 0.2 M, 0.1 M, and 1%, respectively. The samples were incubated at 45°C for 15 min and then extracted at room temperature for 30 min with an equal volume of phenol-chloroform (1:1 vol/vol). After the phases were separated, the top layer was extracted with ether, and the DNA was precipitated with 2 volumes of ethanol in the presence of 0.3 M potassium acetate at −20°C overnight. The precipitate was sedimented by centrifugation at 11,000 rpm for 15 min, suspended in 0.5 ml of 0.1× TEN (1× TEN contains 0.1 M Tris-hydrochloride, pH 8.0, 0.1 M NaCl, and 1 mM EDTA), and dialyzed for 4 h against the same buffer.

Transformation. Competent cells were prepared by the method of Wilson and Bott (31). DNA samples (25 to 50 μl) in 0.1× TEN were added to 100-μl portions of competent cells and incubated with aeration at 35°C for 30 min. The concentration of DNA used was not saturating (less than 0.1 μg/transformation experiment). On completion, the cells were diluted with Wilson-Bott inorganic salts medium and plated onto appropriate selective plates, and *thyB*$^-$ transformants were scored when the donor DNA was *thyB*$^-$ and the recipient was *thyB*$^+$ *thyA*$^-$ (i.e., MS149 or MS154) by selecting for trimethoprim resistance at 35°C. *thyB*$^+$ *thyA*$^-$ strains are thymine requiring and trimethoprim resistant at 45°C but not at 35°C (19). The *ilvA* mutation confers an isoleucine requirement, whereas the *ilvD* mutation confers a requirement for

valine and isoleucine. Thus, the *ilvD*$^+$ phenotype could be scored alone on plates lacking valine or isoleucine. When scoring for *citK* by using strain CU1323 as a recipient, we washed the transformed bacteria in dilution media before plating to avoid carry-over of glucose.

The transforming activity of a DNA in low-gelling-temperature agarose was determined by incubating 10 μl of the melted gel fraction at 35°C with 10 μl of competent cells. After 30 min, the sample was diluted with 100 μl of dilution medium, and 25-μl samples were plated onto appropriate selective plates.

Preparation of lysates. Bacteria to be lysed were cooled and harvested by centrifugation after addition of 1 mM NaN_3. These bacteria were suspended in PM [20% sucrose, 20 mM $MgSO_4$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, 0.02 M Tris-hydrochloride, pH 8.0] at a concentration equivalent to 4 mg (dry weight) per ml and then incubated with 200 μg of lysozyme per ml at 35°C for 30 min. The suspension was cooled to 0°C when protoplast formation was complete, and 3 volumes of ice-cold LM [0.02 M Tris-hydrochloride, pH 7.0, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, 1 mM dithiothreitol, 5 mM $MgSO_4$, 0.05 M NaCl] was added. Ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid was included to inhibit nucleases activated by calcium or manganese (12, 25).

In some experiments, 20 U of restriction endonuclease *Bam*HI per ml was added directly to the lysate. In other experiments (see Table 1) the DNA-membrane complex was sedimented by centrifugation at 10,000 rpm for 10 min, suspended in a mixture of PM and LM (1:3), and then treated with *Bam*HI. The pellet was rapidly dispersed in the presence of excess *Bam*HI. After 1 h, the plasma membrane was sedimented by centrifugation at 8,000 rpm for 10 min. Before the supernatant fraction was applied to sucrose gradients, 0.05 M EDTA was added.

Sucrose gradient fractionation of lysates. The standard conditions used for fractionating lysates by gradient centrifugation were as follows: a 40-ml, 10 to 40% sucrose gradient in 0.5× TEN formed over a 5-ml layer of 76% sucrose in the same buffer. Samples (between 5 and 20 ml) were sedimented by centrifugation at 12,000 to 18,000 rpm for 16 h in a swing-out head (type 3× 65 ml; Measuring & Scientific Equipment, Ltd.). All solutions used for the preparation of lysates and sucrose gradients were sterile. Molecular weights and S values were calculated from the distances sedimented, using phage λ DNA as a 33S standard (4).

Agarose gels. DNAs digested with restriction endonucleases were electrophoresed on vertical 0.8% low-gelling-temperature agarose (thickness, 3 mm; Sigma Chemical Co.). The gels contained 0.09 M Tris–0.09 M borate buffer and 2.5 mM EDTA. The electrode buffer contained the same components, but at half the concentrations; 0.2 volume of 40% sucrose, 0.2 volume of 20% Ficoll, 0.2 volume of 50 mM EDTA, and 0.2 volume of bromophenol blue (1 mg/ml) were added to each sample before the gel was loaded. Electrophoresis was carried out at 24 mA for 16 to 18 h. After electrophoresis the gels were stained with ethidium bromide (1 μg/ml in distilled water) or were soaked in 0.1× TEN for 1 h and then cut into 5-mm slices and stored frozen in sterile glass tubes for subsequent use in transformation experiments. DNA from ethidium bromide-stained gels could not be used for transformation, as 300-nm light inactivated transforming activity.

Use of nitrocellulose filters to isolate complexes containing origin and terminus genes. Specific DNA sequences containing terminus and origin genes were isolated by filtering lysates through nitrocellulose filters (10) as follows. Filters were soaked in hot water (80°C) before use. Dimethyl sulfoxide (final concentration, 5%) was added to the samples to reduce binding of single-stranded tails to the filters (14). A sample (5 to 10 ml) was then drawn through a filter (mounted on a Sweenex plastic filtration unit) at a negative pressure of 8 cm of mercury. Trapped DNA was removed by drawing 10 ml of 0.1× TEN through the filter. The filter was then inverted and eluted with 1% Sarkosyl in 0.1× TEN to remove the bound DNA. The filter was soaked for 5 min in the detergent without any suction. Thereafter, two 5-ml volumes of the Sarkosyl solution were drawn through the filter at a negative pressure of 8 cm of mercury. Between 80 and 90% of the bound DNA was released by this treatment. The remainder was released by soaking the filter in Sarkosyl overnight at 4°C.

The degree of enrichment for terminus and origin markers was greatest when the total amount of cellular material filtered was not greater than 0.1 mg (dry weight) per cm^2. Sartorious and Millipore (type HAWP) filters gave the same enrichment for terminus markers. Similar results were obtained by using filters with pore sizes ranging from 0.22 to 8 μm.

RESULTS

Effect of *Bam*HI on lysate. When protoplasts of *B. subtilis* were lysed by reducing the concentrations of sucrose (20 to 5%) and Mg^{2+} (20 to 10 mM) required for osmotic stability, the viscosity increased as DNA was released. However, all of the DNA was sedimentable into a compact pellet by low-speed centrifugation (10,000 rpm for 10 min). The addition of chelating agents to the lysate caused a further increase in the viscosity of the suspension, and the DNA formed a diffuse fibrous pellet during centrifugation, which was freely accessible to restriction endonucleases. Treatment of lysates by *Bam*HI yielded restriction patterns which were very similar to those obtained when phenol-purified DNA was used (Fig. 2).

The effect of *Bam*HI on the release of DNA and protein from the DNA-membrane complex was examined. Protoplasts were prepared from exponential phase cells that were steady state labeled with $^{35}SO_4^{2-}$ and [^{3}H]thymine.

The protoplasts were sedimented and suspended in PM to remove mesosomal vesicles and extracellular proteins and were then lysed by adding 3 volumes of LM. The lysate was then fractionated into the following three components: material remaining in the supernatant after the DNA-membrane complex was sedimented; material sedimented after *Bam*HI diges-

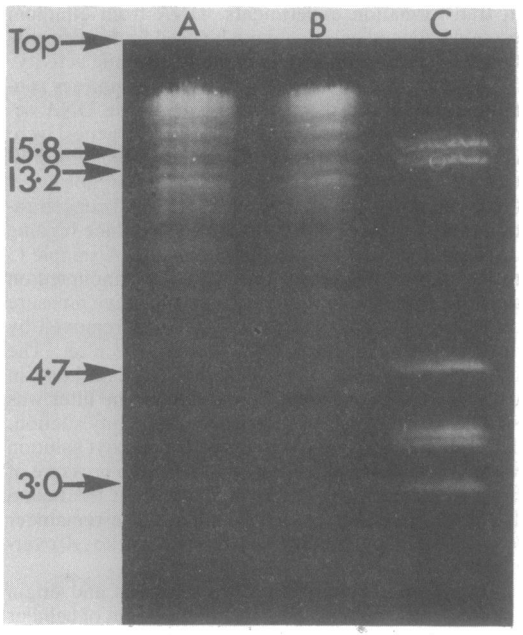

FIG. 2. Electrophoretic separation in 0.8% agarose. Lane A, *B. subtilis* 168 *trpC2 thyA thyB* DNA digested in a lysate with *Bam*HI; lane B, purified *B. subtilis* 168 *trpC2 thyA thyB* DNA digested with *Bam*HI; lane C, phage λ DNA digested with *Eco*RI. Sizes of fragments, $\times 10^6$ daltons.

tion of the DNA-membrane complex; and material released from the complex after *Bam*HI treatment (i.e., in the supernatant after centrifugation) (Table 1). Essentially all of the DNA sedimented with less than 50% of the protein. Almost all of this DNA was released by *Bam*HI treatment. The DNA remaining with the pellet was characterized by DNA-mediated transformation. There was little or no enrichment for any genetic marker in this fraction (data not shown). In similar experiments with germinating spores, about 5% of the DNA remained with the pellet, and this also was not significantly enriched for origin or terminus markers. The DNA found with the pellet may have been from unlysed cells.

TABLE 1. Distribution of protein and DNA after BamHI treatment of protoplast lysate

Prepn	% In:[a]	
	^{35}S-labeled protein	[^{3}H]DNA
Supernatant	52	3
Centrifugal pellet	10	1
*Bam*HI-released material	38	96

[a] Distribution of macromolecules expressed as percentages of the total radioactivity in the sample. Fractions were prepared as described in the text.

Characterization of material released from the pellet complex by *Bam*HI. The material released by *Bam*HI treatment from the fast-sedimenting complex was fractionated on a sucrose gradient (Fig. 3 and 4). The majority of the DNA released by *Bam*HI treatment sedimented in a broad peak with a modal average of 20S. There was also a small but significant amount of fast-sedimenting DNA found in the lower fractions (Fig. 3A). Released protein was found in two major peaks; one of these was at the top of the gradient, and the other was a fast-sedimenting component in fractions 1 through 3 and the sediment (fraction S) (Fig. 3A). A small amount of protein was found in all of the intervening fractions. The high sedimentation coefficient of the DNA in fractions 1 through 5 depended on the association of DNA with other components, as it was not observed after Sarkosyl treatment. Exhaustive digestion of the sample with DNase before fractionation did not affect the sedimentation coefficient of the protein components in fractions 1

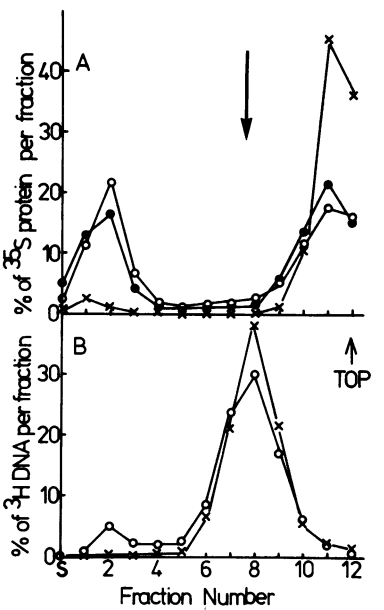

FIG. 3. Proteins (A) and DNA (B) released from a rapidly sedimenting complex by *Bam*HI. Preparations were fractionated on a linear 10 to 40% sucrose gradient in 0.5× TEN and centrifuged at 18,000 rpm for 16 h; the fraction size was 4 ml. The direction of sedimentation was from right to left. Lysate was prepared from strain 168 *trpC2 thyA thyB* in exponential growth in a medium containing $^{35}SO_4$ and [^{3}H]thymine (see text). After preparation of the *Bam*HI-released fraction (Table 1), 0.05 M EDTA was added. Symbols: ●, preparation incubated with 100 μg of DNase per ml for 1 h; ×, preparation heated at 45°C with 1% Sarkosyl for 15 min; ○, no treatment. The arrow indicates the position of phage λ DNA.

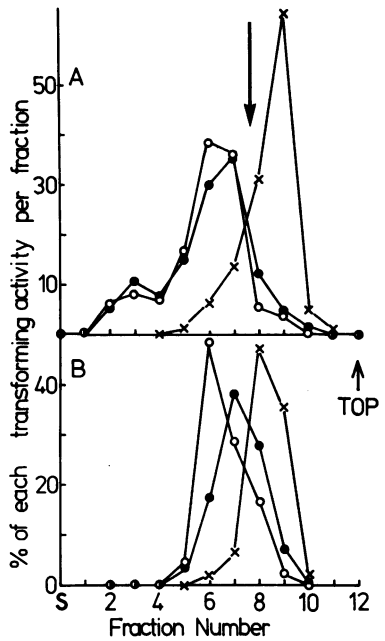

FIG. 4. Fractionation of transforming activities by using DNA from the sucrose gradient described in the legend to Fig. 3. (A) *Bam*HI-digested lysate with no further treatment. (B) *Bam*HI-digested lysate treated with Sarkosyl as described in the legend to Fig. 3. *purA16* (●), *ilvA1* (○), and *hisA1* (×) were determined by using strain MS146 as the recipient.

through 3. However, there was a 30 to 40% reduction in the amount of protein in fractions 4 through 8. This may indicate the presence in this part of the gradient of DNA-protein complexes whose S values were in part determined by their DNA components.

The behaviors of the genetic markers *purA16*, *hisA1*, and *ilvA1* in the gradient in Fig. 3 are shown in Fig. 4. Approximately 20% of the *purA* and *ilvA* transforming activity was found in a distinct peak in fractions 2 and 3, whereas the remainder was found in a large peak of about 50 to 100S. If the sample was incubated at 45°C with 1% Sarkosyl before centrifugation, the fast-sedimenting peak of *purA* and *ilvA* was not observed. However, Sarkosyl had little or no effect on the 50 to 100S peak of both activities. More intensive efforts to deproteinize this sample (by using pronase and phenol) did not significantly reduce the S value of this peak (unpublished data). However, the leading edge of the peak was affected by Sarkosyl (Fig. 4, fraction 5).

The ratio of *purA* transforming activity to *ilvA* transforming activity to *hisA* transforming activity in the sample before fractionation was 2.5:0.5:1.0, whereas in the fast-sedimenting complex (fractions 2 through 4) this ratio was 26:5:1; a purification of 10-fold for both *purA* and *ilvA*. In similar experiments with germinated spores in which the original ratio of *ilvA* to *hisA* was higher, ratios of *ilvA* to *hisA* of 17 have been obtained. Both of these results were obtained reproducibly in a large number of experiments with a number of strains.

The behaviors of 10 genetic markers were studied in *Bam*HI-digested lysates of germinating spores (Fig. 5). The membrane fraction sedimented at low speed, contained approximately 5% of the DNA, and was not significantly enriched for any of 10 genetic markers. This DNA may be present in unlysed bacteria. In the experiment shown in Fig. 5, there was some DNA in a sediment (fraction S). The distinct peak in the lower part of the gradient in Fig. 4A was obscured as larger fractions (8 ml) were collected to reduce the total number of transformation experiments required. However, fractions 2 and 3 were highly enriched for *purA* and *ilvA* compared with *hisA* and were comparable to fractions 2 through 4 in Fig. 4A. The peak of *hisA* in fraction 5 was comparable to fraction 9 in Fig. 4A. In Fig. 5, the data for the other markers examined are arranged with the markers behaving like *purA* on the left and the markers behaving like *hisA* on the right. Markers *thyB* and *ilvD*, which are closely linked to *ilvA*, were broadly similar to *ilvA*.

When purified DNA was used, the frequencies of cotransformation of *thyB* with *ilvD*, *thyB* with *ilvA*, and *ilvD* with *ilvA* were 90, 55, and 40%, respectively (in good agreement with previously published values [2, 3]). These values were not significantly affected by *Bam*HI either when pure DNA was used or in the lysates described above.

The markers surrounding the *ilvA-ilvD* region did not follow the same pattern. The distributions of *kauA1* and *gltA272* were similar to those of *hisA1* and *pheA1*. Similar results were also obtained with *gltA2* (data not shown). The transforming activities of *metB5* and *citK* were greatly reduced by *Bam*HI treatment, and in most experiments these two markers were distributed throughout the gradient (Fig. 5). However, in certain experiments all of the surviving activity was in the upper part of the gradient. The transforming activity of *metB5* relative to *ilvD15* and the linkage of these genes (usually about 50%) was severely reduced by *Bam*HI treatment. When *metB5*$^+$ transformants were selected by using *Bam*HI-treated DNA as the donor, there was some cotransformation of *ilvD15* (data not shown). This suggests that a small proportion of the potential *Bam*HI sites between these markers may remain uncleaved. The *metB5* transforming activity which was still linked with *ilvD15* was found in the fast-sedimenting frac-

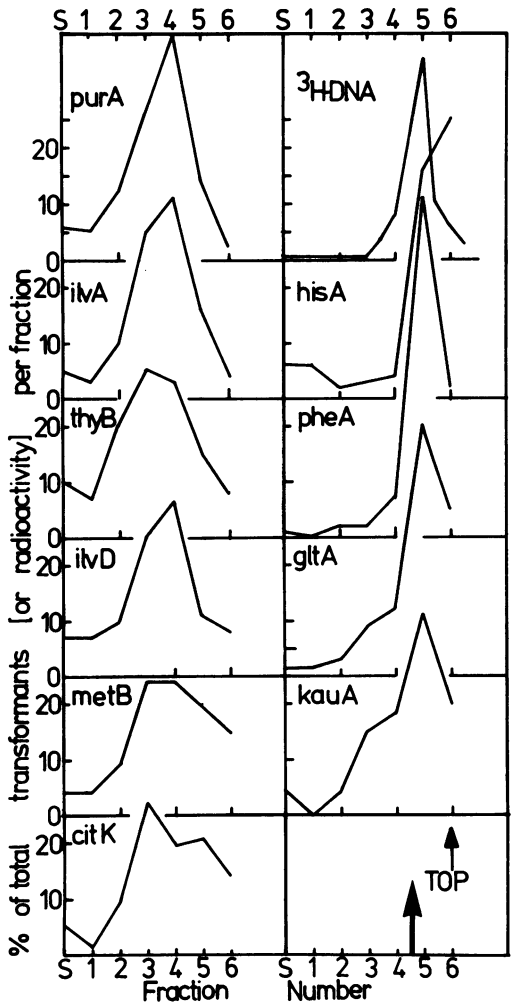

FIG. 5. Sucrose gradient fractionation of transforming activities released by *Bam*HI (Table 1) from *B. subtilis* 168 *trpC2 thyA thyB* growing spores. Gradients (prepared as described in the text) were centrifuged at 12,000 rpm for 16 h (slower than for Fig. 4, to spread the transforming activity more evenly through the gradient). The fraction size was 8 ml. Fraction S was the sediment. The transformation frequencies of markers *purA16*, *ilvA1*, *metB5*, *hisA*, *pheA*, and *gltA292* were determined by using strain MS146, whereas those of *kauA1* and *citK1* were determined by using strain CU1323. Equal volumes of each sample were used for each transformation. Transformation frequencies were expressed as percentages of the total number of transformants obtained from one gradient. The arrow indicates the position of phage λ DNA.

tion enriched for the *ilvA-ilvD* region, whereas the fully digested *metB5* activity was on a considerably smaller fragment and consequently would be expected to have greatly reduced transforming activity (possibly only 20% [17]). As the frequency of *metB5* transformants was similar in the two regions, it seemed likely that the bulk of the *metB* marker cosedimented with *hisA1* and similar markers. The *citK* marker was also very strongly affected by *Bam*HI. The complex behavior of this marker in sucrose gradients suggests that it is close to a particle-bound region, like *ilvA*, from which it may be incompletely cleaved. In the absence of a known linked marker we were not able to clarify this observation.

Experiments similar to those in Fig. 5 but with strain 168 *trpC2* and a strain lacking phage SPβ gave broadly similar results (data not shown).

Location of the prophage of SPβ. The prophage of SPβ is to the right of the *ilvA* gene on the genetic map of *B. subtilis* (Fig. 1). To determine whether SPβ genes were included in the rapidly sedimenting region of the sucrose gradient, a filter hybridization assay (13, 24) was used (Fig. 6). Germinating spores of strain 168 *trpC2* SPβ$^+$ were labeled with [^{3}H]thymidine, lysed, treated with *Bam*HI, and fractionated on a su-

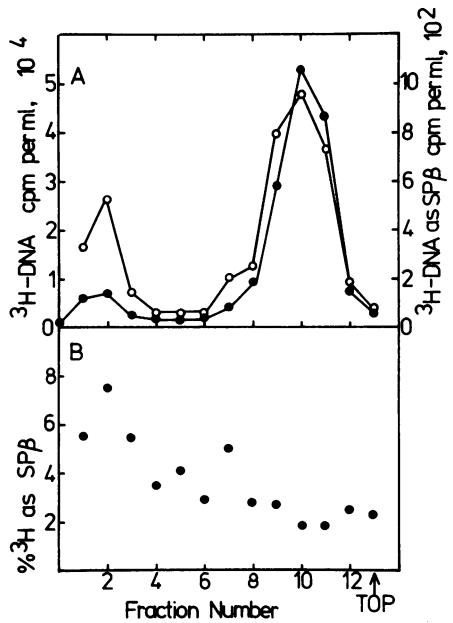

FIG. 6. Distribution of DNA homologous to DNA of SPβ. A *Bam*HI-treated lysate was obtained by using outgrown spores of strain 168 *trpC2* (SPβ$^+$). The spores were steady state labeled with [^{3}H]thymine and germinated in the presence of thymine having the same specific radioactivity (63 Ci/mol). A sucrose gradient was prepared and centrifuged as described in the legend to Fig. 3. (A) Symbols: ●, [^{3}H]DNA in gradient; ○, [^{3}H]DNA in gradient homologous to SPβ$_{cl}$ DNA (determined by using the DNA-DNA hybridization method of Kourilsky et al. [13]). (B) The values are the percentages of [^{3}H]DNA hybridized to SPβ$_{cl}$ DNA filters. SPβ$_{cl}$ is a clear plaque mutant of SPβ (30).

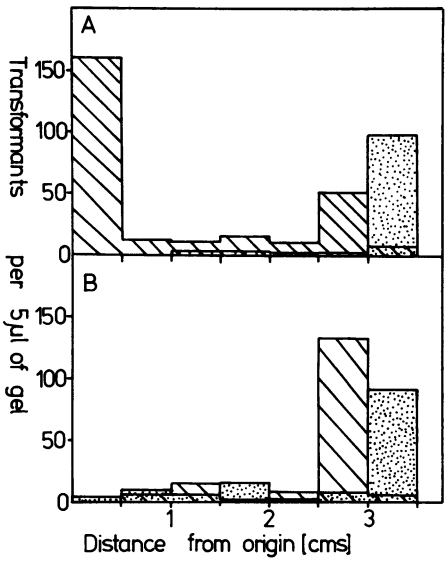

FIG. 7. Electrophoresis on 0.8% agarose of *ilvA* and *hisA* transforming activities from *Bam*HI-released DNA from outgrowing spores. *ilvA* (cross-hatched) and *hisA* (stippled areas) transformants were obtained by using strain MS146 as the recipient. Melted slices of agarose were used for transformation (see text). (A) Untreated sample (200 µl). (B) Sample (200 µl) heated to 45°C with 1% SDS for 20 min before electrophoresis.

crose gradient. Most of the SPβ prophage (70 to 80%) sedimented with the bulk of the DNA in fractions 9 through 11. However, in the lower part of the gradient, where *ilvA* and *purA* were enriched, there was a three- to fourfold enrichment for sequences with homology for SPβ.

Characterization of the *ilvA-ilvD* fragment by using electrophoresis. A large proportion of the *ilvA* and *purA* transforming activity found in *Bam*HI-treated lysates was complexed with material which prevented it from migrating into an agarose gel during electrophoresis. Without sodium dodecyl sulfate (SDS) treatment, most of the *ilvA* transforming activity remained at the origin (Fig. 7), whereas after SDS treatment all of this activity penetrated the gel and migrated with a velocity equivalent to a molecular weight of about 2×10^7, which is considerably less than the molecular weight suggested by the sucrose gradient determination of the molecular weight (10^8) (see above). Without SDS treatment, a small amount of *ilvA* activity was found at the same position as *ilvA* in the SDS-treated gel. The migration of *hisA* transforming activity was not affected appreciably by SDS treatment, and this marker was not found in significant amounts at the origin. *purA* behaved in an almost identical fashion to *ilvA* (data not shown).

The efficiency of DNA-mediated transformation of any marker depends on the size of the double-stranded fragment bearing it (17). DNAs containing *ilvA* from fast- and slow-sedimenting fractions (Fig. 5, fractions 2 and 5) were purified with phenol and electrophoresed on agarose. The migrations of *ilvA* transforming activity from the two fractions were not significantly different. The estimated molecular weight was 1.5×10^7 to 2×10^7.

Retention of origin and terminus markers by nitrocellulose filters. When *Bam*HI-treated lysates were filtered through nitrocellulose membranes, the markers from the *ilvD-ilvA* region and *purA16* were retained by the filter. These were eluted with Sarkosyl (see above) and after purification with phenol were used for transformation studies. Approximately 10% of the DNA in each lysate was retained by the filters, depending on the washing procedure used and the total amount of DNA filtered. Table 2 shows the retention of various markers by filters. *ilvA* and *purA* were strongly retained, whereas *hisA* was able to pass through the filter relatively easily. These origin and terminus markers were not retained by the filters in the presence of Sarkosyl.

Use of specific terminus labeling to study DNA-membrane interactions. Spores which had a [^{14}C]thymine steady-state label and a [^{3}H]thymidine-pulse-labeled terminus (5-min pulse) were prepared as described previously (see above). These spores were germinated, and each culture was harvested after a sixfold increase in optical density. Lysates were treated with *Bam*HI and then fractionated on sucrose gradients (Fig. 8). Only 17.5% of the steady-state label (^{14}C) was found below fraction 6, whereas 43% of the pulse-label was found there. Fractions toward the bottom of the gradient had ratios of ^{3}H to ^{14}C which were three- to fourfold greater than the ratios of the fractions at the top of the gradient. In this experiment, the fast-sedimenting material formed a sediment (Fig. 8, fraction S).

Spores were prepared, steady state labeled with [^{14}C]thymine, and pulse-labeled specifically in the terminal region for 2.5, 5, and 10 min with [^{3}H]thymidine. After germination and outgrowth, lysates were prepared, treated with

TABLE 2. Retention of transforming activities by nitrocellulose filters

Prepn	No. of transformants (10^4)[a]		
	purA	*hisA*	*ilvA*
Filtrate	1.65	1.39	0.39
DNA retained by filter	3.26	0.48	2.39
Unfractionated sample	5.10	3.06	2.26

[a] Number of transformants normalized to the same volume of starting material (100 µl).

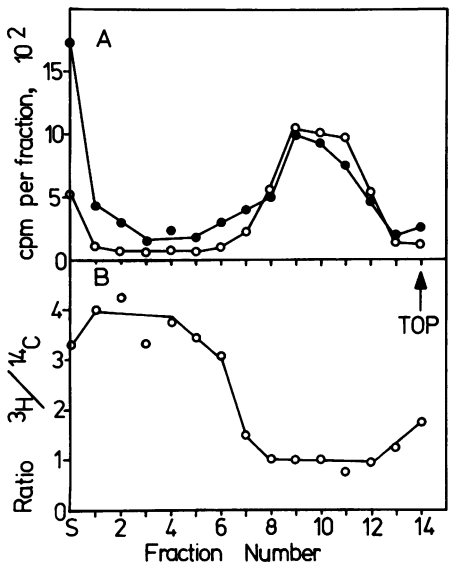

FIG. 8. Density gradient fractionation of *Bam*HI-digested lysate obtained from outgrown spores, pulse-labeled with [^{3}H]thymidine in the terminus (5-min pulse), and steady state labeled with [^{14}C]thymine. A sucrose gradient was centrifuged for 16 h at 18,000 rpm (see legend to Fig. 3). S, Sediment. (A) Symbols: ●, ^{3}H pulse-label; ○, ^{14}C steady state label. (B) Ratio of ^{3}H to ^{14}C.

*Bam*HI, and fractionated as described above (Table 3). The ratios of ^{3}H to ^{14}C in the particulate fraction were greater than those in the bulk fraction by factors of 2.4, 2.9, and 2.0 for 2.5-, 5-, and 10-min pulses, respectively. In independent experiments with longer pulses (20 min), no enrichment was obtained (data not shown).

DISCUSSION

The small amount of DNA remaining with the plasma membrane fraction after *Bam*HI treatment of a lysate was not enriched for origin or terminus markers. This contrasts strongly with the many reports that appear to show attachment of these regions of the chromosome to the plasma membrane after hydrodynamic sheering of the lysate. The DNA remaining in this fraction probably represents a random selection of the DNA sequences that were not resolved by lysis and *Bam*HI treatment. The release of almost all of the DNA, including the origin and terminus regions, from the plasma membrane and a similar observation made after *Eco*RI treatment of *E. coli* lysates (29) could indicate either that DNA is not membrane bound in vivo or that attachment is disrupted under these experimental conditions. We chose lysis conditions that were optimal for preservation of membrane structure and minimized dispersion of the nucleoid but allowed access of restriction endonucleases.

A small proportion of the DNA released was bound to a fast-sedimenting complex which contained protein. We believe that this material is detached from the bulk of the plasma membrane during *Bam*HI treatment. The DNA in this fraction is evidently part of a complex as its sedimentation coefficient is greatly reduced after detergent treatment. However, treatment with DNase did not decrease the sedimentation rate of the protein component of the particle. Phospholipid (labeled with [2-^{3}H]glycerol) was also present in the particle (data not shown). More rigorous proof that this complex is indeed derived from the plasma membrane is not available. However, many of the polypeptides observed in this fraction appeared to correspond to a subset of the proteins of the plasma membrane when they were examined by two-dimensional gel electrophoresis (unpublished data).

The DNA in this fraction was highly enriched for specific genes from the chromosomal origin and terminus, and these also were released by Sarkosyl treatment. The particles contained about 20% of the total *purA* and *ilvA-ilvD* transforming activities, and these activities were purified about 10-fold compared with *hisA*. The enrichment for terminus markers in this fraction was greater than any previously described particulate terminus-complex and concerned a smaller region of the chromosome.

The remainder of the *ilvA-ilvD* and *purA* transforming activities were found in a broad peak of 50 to 100S. The leading edges of the *ilvA* and *purA* peaks were clearly sensitive to Sarkosyl. This indicates that there may be a range of particles, containing varying amounts of protein, intermediate between the fast-sedimenting material and the 50S peak.

When freshly prepared *Bam*HI-treated lysates were electrophoresed on agarose or drawn through nitrocellulose filters, a large proportion of the *ilvA* or *purA* transforming activity could not penetrate the agarose or was retained by the

TABLE 3. Effect of pulse length on labeling of the particulate fraction[a]

Prepn	^{3}H/^{14}C ratios after the following pulse lengths		
	2.5 min	5.0 min	10 min
Particulate fraction	0.19	0.53	0.73
Remainder	0.08	0.18	0.36
Total DNA	0.1	0.24	0.43

[a] Ratios of ^{3}H to ^{14}C in fractions prepared and labeled as described in the legend to Fig. 8. The particulate fraction contained about 20% of the total (fractions S and 1 through 6).

filters. These effects were not observed if the samples were first treated with a detergent. This suggests that a substantial proportion of the *ilvA* and *purA* activities were in a complex.

The reason why DNA should be totally released from the bulk of the membrane remains obscure. We suggest that once sufficient cleavages have occurred, the DNA is free to move, to interact with solvent, and to pull the attachment region out of the membrane.

The behavior of DNA labeled in the terminus region isolated from germinating spores was examined. A substantial part of the pulse-label was fast sedimenting. If the precise replicative terminus was the actual point of attachment to a membrane complex, then the DNA-membrane complex of cells derived from spores that had been terminus labeled for short times would be highly enriched in the membrane complex. The maximum enrichment for pulse-label in the fast-sedimenting complex was with a pulse of 5 min, although enrichment was substantial even at 10 min. The effect of pulse length on the labeling of the DNA-membrane complex can be explained in two ways; either the bound region of the chromosome is broad, or the bound region lies to one side of the terminus. Our analysis of the markers enriched in the fast-sedimenting complex supported the latter hypothesis. Thus, *gltA*, which is probably closest to the replicative terminus (6), is not part of the complex, whereas the *ilvA-ilvD* cluster is probably separated from *gltA* by about 6% of the chromosome. The lack of association of most of the prophage of SPβ with the complex indicates that much of the intervening chromosome is not part of the complex either. Other attachment regions (perhaps in the vicinity of *citK*) may be possible.

There is still no positive evidence indicating that the terminal region of procaryotic chromosomes has a regulatory role during growth and division. There appears to be a fixed terminus in *E. coli* between *rac* and *man* (31 and 35 min, respectively) even when replication is initiated from another site (16). On the other hand, there are strains of *B. subtilis* which grow normally with large deletions in the terminal region (8). S. A. Zahler (personal communication) has isolated strains in which deletions extend from the left end of SPβ to beyond *gltA* as a result of excision of SPβ. Merodiploids have been isolated in which a copy of the *metB-ilvA* region has been inserted at a point between *hisA* and *thr* (Fig. 1), whereas density transfer studies have clearly shown that *citK* is still the last marker to replicate in these strains (28). Rosenthal et al. (21) have shown that *B. subtilis* cells can also have two copies of parts of the terminal region of the chromosome when they are lysogenized with specialized defective transducing phages. Strains diploid for the *ilvA-ilvD* region have been isolated. It appears that the known structural features of the terminal region can be rearranged genetically without adversely affecting the growth process.

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