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Identification of Specific Restriction Fragments Associated with a Membrane Subparticle from *Bacillus subtilis*

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When lysates of *Bacillus subtilis* were treated with restriction endonucleases *Eco*RI or *Hin*dIII, almost all of the DNA was released from the major plasma membrane fraction that was sedimentable at low speed. However, a very small part of the released DNA, when centrifuged at high speed, appeared to be bound to small membrane fragments. On agarose gels, this material, prepared with either enzyme, contained only a small number of restriction fragments, and the DNA in the sample hybridized with 11 to 12 *Eco*RI or *Hin*dIII fragments of chromosomal DNA. This DNA was used after nick-translation to screen Charon 4A clone banks for phages containing membrane-bound fragments. One of these was studied in detail. Only a part (about 5 kilobases) of the region present in this clone is important in binding the DNA to the membrane subparticle.

The attachment of DNA to membranes of bacteria has been extensively studied by genetic transformation and specific labeling methods (12, 21) but has rarely been studied at the level of restriction fragments. Specific restriction fragments from the origin region of *Escherichia coli* have been identified in the outer membrane fraction prepared after mechanically disrupting the bacteria, although DNA from other parts of the chromosome was also present in this fraction (14, 15, 26). In contrast, when membrane-bound nucleoids of *E. coli* were digested with restriction enzymes, the DNA remaining associated with the membrane appeared to be nonspecific (4).

Restriction enzymes can also be used to dissect the bacterial chromosome with minimal damage to the membrane and to give precisely defined DNA fragments. Valenzuela and colleagues (23, 24) have shown that restriction enzymes release almost all of the DNA from the cell envelope of *E. coli* and *Bacillus subtilis* (prepared by high-speed centrifugation) but that the small amount remaining is substantially enriched for replication forks and the origin region. Similar observations have been made with *Bam*HI-treated lysates of *B. subtilis* (21). The bulk of the protoplast ghost was centrifuged out at low speed with very little DNA attached, and this DNA had no apparent specificity. However, a particle-bound DNA fraction could be sedimented from the supernatant at high speed. This fraction was highly enriched for the origin marker *purA* and the terminus markers *ilvA*, *ilvD*, and *thyB* but did not include the neighboring markers *metB*, *gltA*, and *kauA* (21).

In this communication, we used *Eco*RI and *Hin*dIII, which make more frequent breaks than *Bam*HI, and obtained a more specific particle-bound DNA fraction that has proved useful as a probe for clones containing membrane-bound DNA. Using the two enzymes, we were able to determine more precisely where, within a specific region, the attachment region is located.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and radioactive labeling. *B. subtilis* 168 *trpC2* was grown in the low-sulfate medium described previously (21). Lysates were prepared from bacteria harvested at an optical density (at 540 nm) of 2.0 after more than 10 generations of exponential growth. DNA was labeled by adding [^{3}H]thymidine (1 μCi/ml; specific radioactivity, 52 Ci/mmol) at an optical density of 0.8. Phospholipids were labeled with [2-^{3}H]glycerol as described previously (20). [2-^{3}H]glycerol is incorporated into both phospholipid and teichoic acid. However, after lysozyme digestion of the cell wall, only phospholipids are precipitated with trichloroacetic acid.

Purification of DNA. DNA was purified with phenol. Samples from sucrose gradients were dialyzed exhaustively in the presence of Sarkosyl and EDTA at 2°C before further purification.

Preparation of lysates, treatment with restriction endonucleases, and sucrose gradient fractionation. Lysates prepared from 50 ml (21) of culture at an optical density (at 540 nm) of 2.0 (100 mg [dry weight]) were treated with 200 U of *Eco*RI or *Hin*dIII and fractionated on a 10 to 40% sucrose gradient with a 76% shelf, as described previously (21). Sodium dodecyl sulfate was

added to samples from sucrose gradients before electrophoresis.

DNA-DNA hybridization. Samples in agarose gels were partially depurinated (25), transferred to sheets of nitrocellulose (22), baked under vacuum for 2 h at 80°C, and prehybridized for at least 16 h (19).

For quantitative DNA-DNA hybridization, all of the samples (from the sucrose gradients) were applied to the same sheet of nitrocellulose (marked with a grid drawn in soft pencil). The samples were denatured by heating at 98°C for 20 min and cooled in ice. Sodium chloride was added to give 1 M. The samples were then drawn onto the nitrocellulose filters (wetted with 0.1% sodium dodecyl sulfate) under negative pressure. After the samples were loaded, the filters were laid (with the sample uppermost) on filter papers soaked in 0.5 M sodium hydroxide and 1 M sodium chloride for 3 min and then transferred to a pad of dry blotting paper and subsequently to a pad soaked in 1 M sodium chloride and 0.5 M Tris-hydrochloride (pH 7.5). The filters were baked and prehybridized as described above.

Hybridization was carried out by the method of Kourilsky et al. (10) with 3× SSC (1× SSC, 0.15 M NaCl plus 0.015 M sodium citrate) for 24 h (19). Autoradiographs of the dried filters were prepared with prefogged X-ray film and intensifying screens (11). Filters for quantitative hybridization were cut up after the washing procedure and counted in Packard scintillation fluid.

Isolation of Charon 4A clones containing membrane-bound DNA. Plaques of the Charon 4A clone bank of Ferrari et al. (6) were transferred to nitrocellulose filters and screened for homology with probes prepared from the fast-sedimenting DNA fraction (see below; 2). Isolated phages were subsequently amplified and purified as described by de Wet et al. (3).

Nick-translation. Samples of DNA were nick translated to high specific activity (9).

Electron microscopy. Samples from density gradients were diluted into 1 M ammonium acetate (pH 7.0), mixed with cytochrome *c* (to 100 μg/ml), and spread onto 0.1 M ammonium acetate, pH 7.0. Occasionally, 0.5% (vol/vol) formaldehyde was added to both the spreading solution and the hypophase. Carbon-coated grids (400 mesh; Cu/Rh) were touched to the surface of spread preparations, stained with uranyl acetate (7), and rotary shadowed with platinum at an angle of 8 to 10°. Micrographs were taken at 60 kV on a JEOL JEM 100CX electron microscope or with a Philips EM300. The magnification of both microscopes was calibrated with the replica of a diffraction grating (2,160 lines per mm). Contour lengths of spread molecules were measured with a Kueffel and Esser map measurer.

RESULTS

Effect of *Eco*RI and *Hin*dIII on lysates. After the treatment of the lysates of *B. subtilis* with *Eco*RI or *Hin*dIII, the cell membrane fraction could be sedimented at low speed (10,000 rpm for 10 min) with very little DNA attached. As in a previous report (21), in which other restriction endonucleases were used, less than 3% of the cell DNA sedimented with this fraction, and it had no discernable sequence specificity. The released material was fractionated by sucrose gradient centrifugation. About 1% of the DNA released by either enzyme was present in fractions 1 to 4, substantially in advance of the bulk of the DNA. The addition of more enzyme to the lysate did not significantly decrease the amount of DNA in this fraction or change the pattern of the DNA bands seen in agarose gels.

The DNA in the fast-sedimenting fraction appeared to be part of a substantial structure. If the lysate was treated with a detergent before centrifugation, a fast-sedimenting component was not observed (data not shown; 21). Furthermore, the DNA component was not significantly larger than the DNA in the major DNA band (data not shown). The fast-sedimenting DNA cosedimented with a substantial amount of phospholipid. This was shown with [2-^{3}H]glycerol-labeled bacteria (see above). Approximately 74% of the ^{3}H-labeled phospholipid was found in the low-speed centrifuged pellet. When the supernatant was fractionated in sucrose gradients (Fig. 1B), phospholipid was found throughout the gradient. A total of 70% was in fractions 1 to 6 where the detergent-sensitive DNA fraction was found.

Electron microscopy of spread fractions. Under electron microscopy, the DNA molecules in the fast-sedimenting fraction contained one or two small electron-dense irregularly shaped structures (Fig. 2A and B). These were judged to be membrane fragments from their similarity to the material seen in preparations of burst protoplasts. Occasionally, a network of DNA fibrils was formed to several particles (Fig. 2A). The slow-sedimenting fractions contained DNA which was not associated with membrane fragments (Fig. 2C). The average maximum dimension of 43 membrane fragments (0.097 ± 0.055 μm) was less than one-tenth of the protoplast ghost. DNA associated with membrane pieces often appeared as rosettes (in spite of variations in the ionic strength of the spreading solutions; data not shown), which precluded an accurate determination of the contour length.

Electrophoretic analysis of DNA present in the particulate fraction. Although only a small amount of particle-bound DNA could be isolated, we could see discrete bands after electrophoresis. Some of these were partial digestion products as the pattern simplified after redigestion of the isolated DNA (Fig. 3). The most prominent bands in the *Eco*RI preparation were 8.5 and 11.5 kilobases (kb), and in the *Hin*dIII preparation they were 10.2, 6.4, and 4.3 kb. By nick-translated particle fraction DNA, we could probe electrophoretically separated restriction fragments of chromosomal DNA (Fig. 4). The patterns obtained with either probe, against

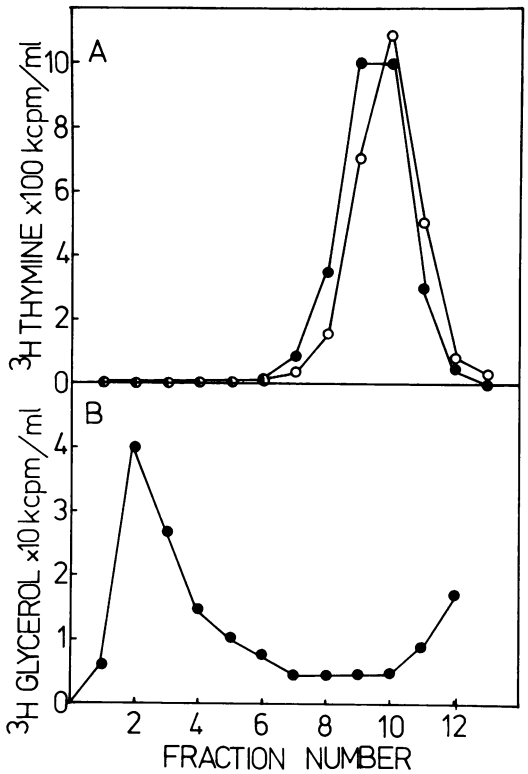

FIG. 1. Fractionation of restriction enzyme-treated lysates on sucrose gradients. The lysates were prepared and treated with restriction enzymes as described in the text. On completion, plasma membrane was removed by low-speed centrifugation. EDTA (0.05 M) was added to the supernatant, which was layered on a 10 to 40% sucrose gradient and centrifuged for 20 h at 20 krpm. The tube washings are in fraction 0. The direction of sedimentation is from right to left. (A) [^{3}H]thymidine-labeled DNA. Symbols: ○, *Hin*dIII-digested lysate; ●, *Eco*RI-digested lysate. The gradients contain approximately 1.1×10^7 cpm of [^{3}H]thymidine. (B) ^{3}H-glycerol-labeled phospholipid in *Eco*RI lysate. The gradients contain approximately 3×10^6 cpm.

DNA digested with *Hin*dIII, were almost identical and contained 11 bands. When the *Eco*RI particle fraction was used against *Eco*RI-digested DNA, 12 bands were seen, but two (12.2 and 1.2 kb) were absent when *Hin*dIII-digested DNA was used against *Eco*RI particle fraction (Table 1). The Southern blot analysis provides more information than do agarose gels, especially in the lower size range where nothing was visible. In general, the same sequences were particle bound when they were prepared with either enzyme. This was corroborated by the high level of homology between particle fraction DNA prepared with either enzyme (unpublished data). The bands visible in agarose gels were identifiable in the Southern blot (Table 1), but their relative intensities did not appear to match exactly.

Isolation of clones containing particle-bound DNA attachment regions. We used the particle fraction DNA after nick translation to screen a clone bank (6) for homologous sequences. Several isolates were obtained which are greatly enriched in membrane fractions. The best of these (φ529) was used in this study to illustrate the behavior of a clearly defined sequence during the procedures used. The relative amount of DNA with homology for φ529 in the gradient fractions (Fig. 5A and B) was determined by the quantitative hybridization method described above. The values for fractions 1 to 4 of either gradient were high, whereas a reduced but significant hybridizing activity was seen in the upper part of the gradient. As a control, another phage (φ1023), isolated from the clone bank, was used in a parallel set of experiments. DNA homologous with this phage was confined almost entirely to the region where the bulk of the DNA sediments and was not found in the lower part of the gradient.

A more precise study of the attachment region homologous with φ529 was possible by the Southern transfer procedure and DNA-DNA hybridization. To facilitate interpretation, we prepared a restriction map of the phage by standard methods. The cloned DNA contains four *Eco*RI fragments (Fig. 6). The fragments

TABLE 1. Sizes of restriction fragments prepared from chromosomal DNA with *Hin*dIII and *Eco*RI hybridizing with particle fraction DNA

*Eco*RI			*Hin*dIII		
Size of fragment (kb)	Probe[a]		Size of fragment (kb)	Probe[a]	
	E_P	H_P		E_P	H_P
12.2[b]	+	−	11.2[b,c]	+	+
8.3[b]	+	+	5.7[b]	+	+
6.2	+	+	5.4	+	+
3.85	+	+	5.0	+	+
3.6[c]	+	+	4.4[b]	+	+
3.25	+	+	3.0	+	+
3.1	+	+	2.8	+	+
2.8[c]	+	+	2.6	+	+
2.6	+	+	1.95	+	+
1.25	+	−	1.1	+	+
1.1	+	+	1.0	+	+
0.8	+	+			

[a] The probe was nick-translated particle fraction prepared with H_P or E_P. + and −, Presence or absence, respectively, of the band shown.

[b] Probably corresponds to bands on agarose gels (Fig. 3).

[c] Possibly present in φ529.

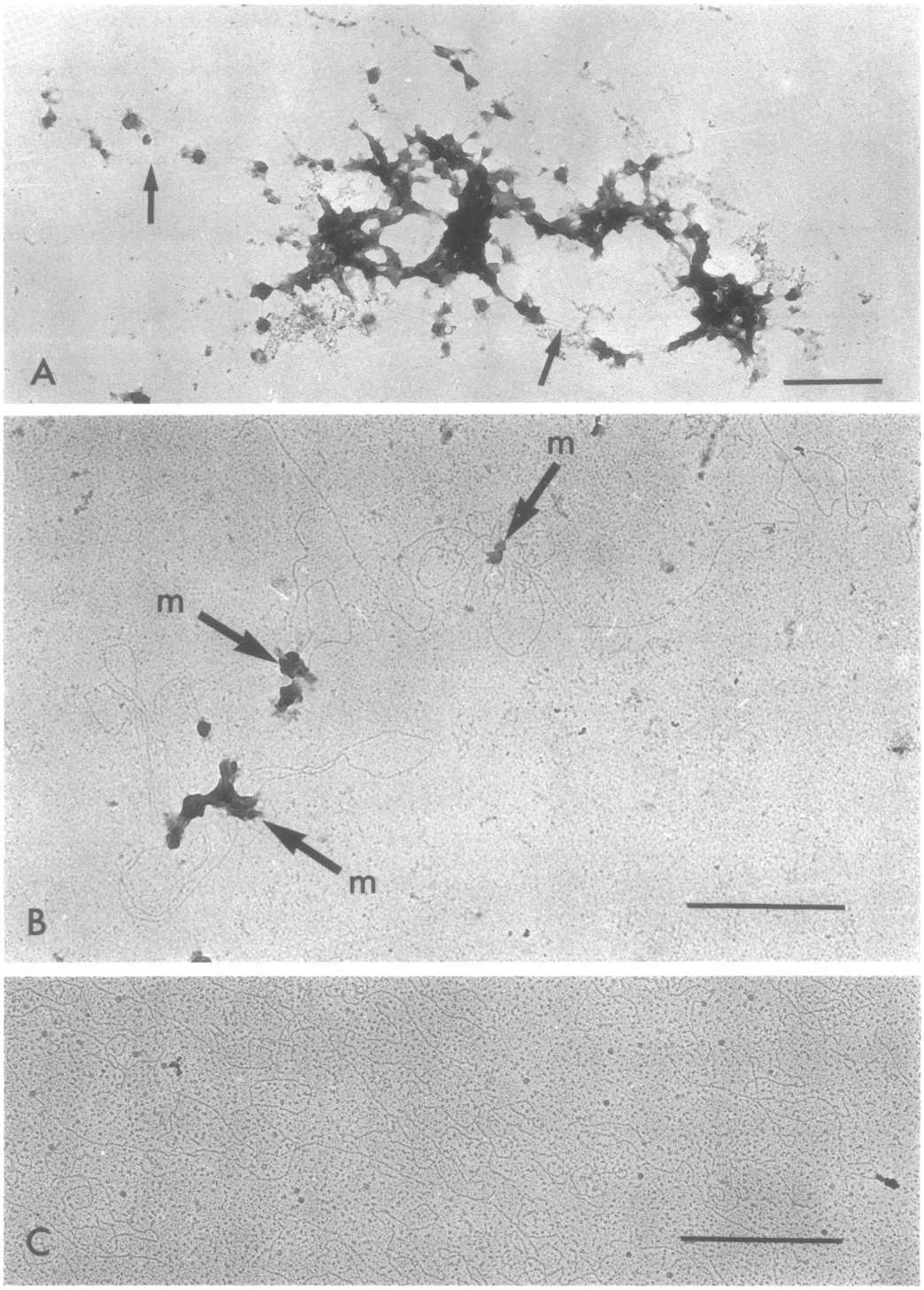

FIG. 2. Spread preparations showing DNA-membrane complexes from fraction 2 (Fig. 1). (A and B) m, Membrane fragments; the arrows show DNA fibrils in (A). (C) Fraction 10 (Fig. 1) contained no trace of membranes. The bar marker equals 0.5 μm.

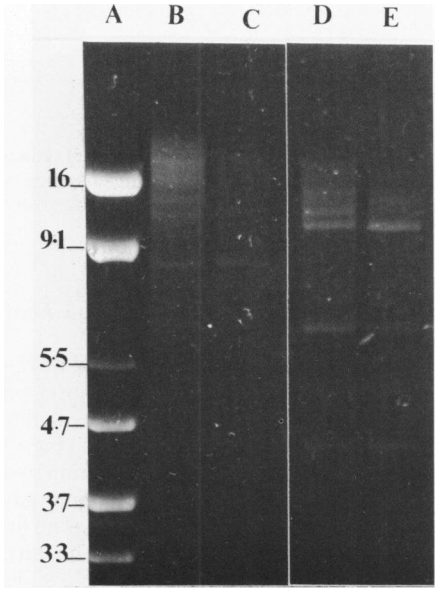

FIG. 3. Agarose gel electrophoresis of particle fraction DNA. Track A, Standard λ DNA digested with *Eco*RI and *Bam*HI (the fragment sizes are given in kb). Track B, Particle fraction prepared with *Eco*RI; track C, particle fraction prepared with *Eco*RI and purified DNA redigested with *Eco*RI; track D, particle fraction prepared with *Hin*dIII; track E, particle fraction prepared with *Hin*dIII and purified particle fraction DNA redigested with *Hin*dIII.

derived from the phage with *Eco*RI, *Hin*dIII, or both enzymes have been named E, H, or EH, respectively (a lower case letter indicates the order of the fragments from the left-hand side). The order was unambiguously established by isolating the large *Hin*dIII and *Xba*I fragments which include the vector and then digesting them with *Eco*RI (data not shown). Furthermore, in samples partially digested with *Eco*RI, we repeatedly observed fragments Eab and Ecd. The sizes of the *Hin*dIII fragments flanking the cloned region in the chromosome were determined by the hybridization of the cloned DNA with *Hin*dIII-digested chromosomal DNA. Thus, the ends of the cloned region are located in 5.35- and 12.5-kb fragments at the left and right ends respectively (Fig. 7, track n). The hybridization of the nick-translated phage with chromosomal DNA digested with *Xba*I or *Hin*dIII also showed that the internal fragments generated by *Xba*I or *Hin*dIII from the phage were also present in the chromosomal DNA. This strongly suggested that the order of *Eco*RI fragments in the phage is the same as that in chromosomal DNA and that rearrangement had not occurred in the construction of the phage.

Analysis of particulate fraction DNA with a second restriction enzyme. Although enzymes were used in excess in the preparation of particulate fractions, the φ529 region was relatively resistant to *Eco*RI or *Hin*dIII. This may indicate that there was some measure of protection of cleavage sites by membrane fragments. To analyze further the attachment region of the φ529 sequence, we prepared particle fraction or free DNA, using *Eco*RI or *Hin*dIII (referred to as E_P, H_P, E_F, or H_F, where E and H indicate the enzyme used and the subscripts P or F are particle or free DNA, respectively). Particle fraction DNA was obtained from fractions 1 to 4, and free DNA was obtained from fractions 8 to 10 (Fig. 1). To establish which parts of the cloned region were present in the particle fraction, we redigested DNA from the latter with the enzyme used in its preparation and identified the fragments with homology for the phage by hybridization (Fig. 7). This was corroborated by double digestion with *Eco*RI and *Hin*dIII (Fig. 7 and Table 2).

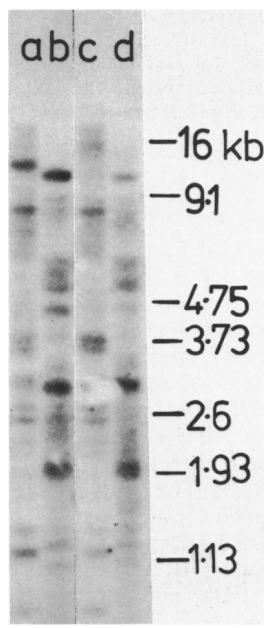

FIG. 4. Hybridization of particulate fraction DNA with chromosomal DNA digested with *Eco*RI (tracks a and c) or *Hin*dIII (tracks b and d). Digested DNA was electrophoresed on agarose, transferred to nitrocellulose, and hybridized with nick-translated particle fraction DNA. Tracks a and b were hybridized with E_P. Tracks c and d were hybridized with H_P. The calibration was obtained from phage λ digested with *Bam*HI and *Eco*RI. The sizes of the fragments are given in kb.

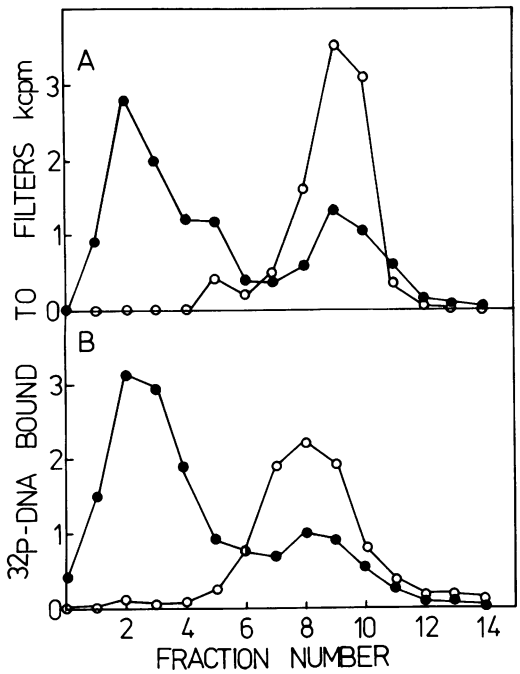

FIG. 5. Distribution of DNA with homology for ϕ529 and ϕ1023 in sucrose gradients. Lysate preparation and centrifugation conditions were as described in the text or the legend to Fig. 1. DNA-DNA hybridization was carried out with 50-μl samples from gradients as described in the text. (A) *Eco*RI-treated lysate; (B) *Hin*dIII-treated lysate. Symbols: ●, ϕ529; ○, ϕ1023.

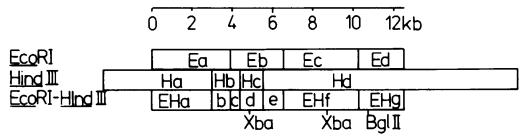

FIG. 6. Restriction map of ϕ529. The fragments generated by *Eco*RI, *Hin*dIII, or both enzymes are designated E, H, or EH. See Table 1 for the sizes of fragments.

Untreated E_P and E_F (Fig. 7, tracks a and d) contained a number of bands between 9 and 12 kb which were larger than any ϕ529 fragment. These were partial digestion products, which disappeared when redigested with *Eco*RI (Fig. 7, tracks b and e). When compared with total chromosomal DNA hybridized with ϕ529, E_P was deficient in the 3.9- and 2.2-kb fragments (i.e., Ea and Ed, respectively), whereas E_F had all four fragments. This was confirmed by an additional digestion with *Hin*dIII. E_P (Fig. 7, track c) contained strong 3.6- and 1.0-kb fragments (EHe and EHf, respectively) and very weak 2.85-kb (EHa) and 2.1-kb (EHg) bands. The positions of these fragments in the map of ϕ529 indicated that the attachment region was in the center of the cloned region (Fig. 6). All of the fragments which could be generated from ϕ529 by double digestion with *Eco*RI and *Hin*dIII were present in E_F (except for EHc, which was not observed under any circumstances). In this experiment, there was an abnormal amount of the ϕ529 region released from the particle fraction during preparation. However, the binding region clearly spanned the two internal *Eco*RI fragments (Eb and Ec) of ϕ529.

Untreated H_P contained a heavy band of 12 to 14 kb (Fig. 7, track g) which, on redigestion with *Hin*dIII, gave bands of 12.5 and 1 kb which we believe to be Hd and Hc, respectively (Fig. 7). This was confirmed by double digestion with *Eco*RI-*Hin*dIII (Fig. 7, track i), which gave strong bands at 3.6, 2.2, and about 1 kb. The first two correspond to EHf and EHg. The latter fragment was presumably EHd and EHe super-

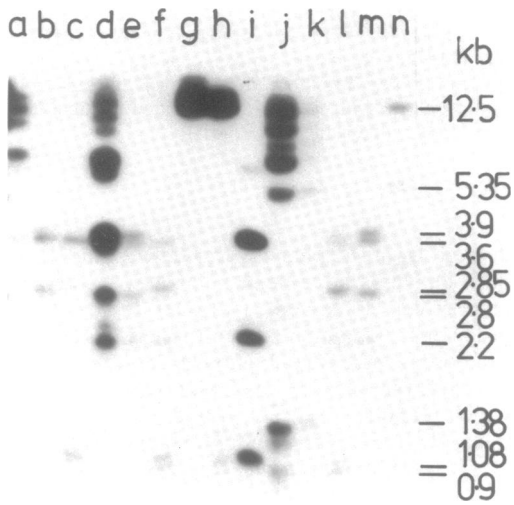

FIG. 7. Hybridization of particle fraction DNA with ϕ529. E_P and H_P were prepared from fractions 1 to 4 of sucrose gradients similar to those in Fig. 1. E_F and H_F were prepared from fractions 8 to 10. After purification with phenol, this material was separated electrophoretically on agarose after redigestion with restriction enzymes as shown for each track. Track a, E_P untreated; track b, E_P + *Eco*RI; track c, E_P + *Eco*RI + *Hin*dIII; track d, E_F untreated; track e, E_F + *Eco*RI; track f, E_F + *Eco*RI + *Hin*dIII; track g, H_P untreated; track h, H_P + *Hin*dIII; track i, H_P + *Eco*RI + *Hin*dIII; track j, H_F untreated; track k, H_F + *Hin*dIII; track l, H_F + *Eco*RI + *Hin*dIII; tracks m and n, chromosomal DNA digested with *Eco*RI and *Hin*dIII, respectively. The size calibration is shown in kb.

TABLE 2. Sizes of restriction fragments hybridizing with ϕ529 in particle and free fractions prepared with *Eco*RI and *Hin*dIII

ϕ529 fragment	Size[a] (kb)	E_P[b]	E_F[c]	H_P[d]	H_F[e]
Ea	3.9	+	++		
Eb	2.8	++	++		
Ec	3.6	++	++		
Ed	2.2	−	++		
Ha	5.35			−	++
Hb	1.38			−	++
Hc	1.08			++	−−
Hd	12.5			++	−−
EHa	2.85	(−)	++	−	++
EHb	0.9	−	++	−	++
EHc	0.45	?	?	?	?
EHd	1.08	(−)	++	++	−
EHe	1.0	++	++	+	−
EHf	3.6	++	++	++	+
EHg	2.2	(−)	++	++	+

[a] The sizes of the chromosomal fragments homologous with ϕ529 are given (see the legend to Fig. 6 for nomenclature).

[b] Figure 1, fractions 1 to 4. ++, Strong; +, less strong; − or (−), absent or very weak band corresponding to ϕ529 fragments of the size shown.

[c] Figure 1, fractions 8 to 10.

[d] Figure 1, fractions 1 to 4.

[e] Figure 1, fractions 8 to 10.

imposed. The complete absence of a 2.8-kb fragment (i.e., EHa) confirmed that the left-hand side of the cloned region was not present in the particle fraction. Partially digested DNA homologous with the ϕ529 region was seen in fraction H_F (Fig. 7, track j). The redigestion of this material with *Hin*dIII (Fig. 7, track k) generated fragments of 5.35 and 1.38 kb (Ha and Hb, respectively) but not a 1.08-kb fragment corresponding to Hc. This suggested that Hc was also particle bound but that regions to the left were not. The double digestion of H_F with *Eco*RI and *Hin*dIII gave fragments corresponding to EHa and EHb, which would be expected from the restriction map (Fig. 6). It seems likely, therefore, that the particle-bound section of the ϕ529 region includes EHd, e, and f. The distribution of the restriction fragments of ϕ529 between fractions P and F is summarized in Table 2.

DISCUSSION

Membrane-bound DNA sequences with a specificity defined at the level of restriction fragments have rarely been described. However, fragments from the origin have been identified in the *E. coli* outer membrane (14, 15, 26) and in a less clearly defined particulate fraction from *B. subtilis* (27). We have now demonstrated that specific restriction fragments associated with a fast-sedimenting complex can be isolated from lysates of *B. subtilis* after restriction enzyme treatment. Particle-bound DNA prepared with either *Eco*RI or *Hin*dIII has considerable homology and hybridizes with the same fragments in chromosomal DNA; it therefore includes common sequences which mediate association with the membrane. It is now possible to clone these fragments and to determine genetically their exact location on the chromosome without relying on the availability of mutations in the membrane-associated region (21). We were unable to find clones containing all of the fragments in the particle fraction. However, we isolated several clones which are highly enriched in the particle fraction. The best available at present (ϕ529) was used to study the fractionation procedure. More than 60% of the DNA homologous to this phage is present in the particle fraction, which contains about 1% of the total DNA. The fragments in the particle fraction which correspond to the ϕ529 region are probably identifiable (Table 1). The study of the ϕ529 region has revealed other aspects of the organization of a particle-bound region. Thus, the binding region can shield certain restriction sites (i.e., between Hc and Hd), or two contiguous fragments can be particle bound (i.e., Eb and Ec), although the intervening site is sensitive to the enzyme.

Most earlier work suggests that there may be many sites of attachment of the chromosome to the membrane, in addition to the origin, terminus, and replication fork regions (12). The number of attachment regions was calculated from data obtained in experiments in which the size and amount of membrane-bound DNA were determined after making specific numbers of double-strand breaks (1, 5, 8, 18). One report has suggested that the chromosome may be attached to the membrane by no more than three sites, of which the origin and replication forks are two (13).

By using restriction enzymes to dissect the nucleoid, we reached a view of chromosome attachment which contrasts strongly in two respects with this widely held view. The first difference is that, whereas the membrane-bound DNA used by previous workers unequivocably involves the entire plasma membrane, we found specific restriction fragments associated with what appears to be only a small fragment of the membrane, which may represent small but specific sites to which the chromosome is attached. Networks of DNA filaments associated with several pieces of membrane (Fig. 2) suggest that one membrane fragment may be attached to the chromosome at a number of points although these may be from one part of the chromosome.

We have suggested previously that restriction

enzyme cleavage of the chromosome in a lysate may cause the chromosome, which is normally constrained in domains of supercoiling in the nucleoid, to uncoil explosively and pull attachment regions out of the membrane (21). Each DNA fragment observed in the particle fraction may, therefore, be attached to membrane subparticles.

The second difference is that the number of attachment sites indicated by these experiments is less than the minimum estimates in previous work (1, 5, 8, 18). The maximum estimate from our data would be the number of completely digested DNA fragments present in the particle fraction. However, this is likely to be an overestimate as some of these may be contiguous in one attachment region, and some may be of minor importance. An explanation of this discrepancy is suggested by a recent reexamination of procedures for calculating the number of attachment sites (16). In addition to fragments containing genuine membrane attachment regions, there could be a network of fragments which are part of the nucleoid core, bound or trapped in a membrane-DNA complex.

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