JOURNAL OF BACTERIOLOGY, Feb. 1985, p. 589–595
0021-9193/85/020589-07$02.00/0
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Vol. 161, No. 2

Amplification of a Major Membrane-Bound DNA Sequence of *Bacillus subtilis*

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Received 27 June 1984/Accepted 16 October 1984

A membrane-bound DNA sequence from *Bacillus subtilis* was subcloned into a plasmid which can replicate in *Escherichia coli* but not in *B. subtilis*. This plasmid hybridized with an 11-kilobase *Hin*dIII fragment which is the major particle-bound fragment in lysates treated with *Hin*dIII. The plasmid integrated into the *B. subtilis* chromosome at the region of homology, conferring chloramphenicol resistance on the recipient. The inserted resistance was mapped close to *purA* by using the generalized transducing phage AR9. In one chloramphenicol-resistant strain, the pMS31 region was repeated at least 20 times. A large proportion of the copies of the cloned region were present in the particle fraction, indicating that the capacity to bind this region of the chromosome was substantially in excess of the normal dose of the region. The structure of the particle-bound region was sensitive to ionic detergents and high salt concentrations but was not greatly affected by RNase or ethidium bromide. The basis of a specific DNA-membrane interaction can now be studied by using the amplified region, without the complications of sequences required for autonomous plasmid replication.

The replication forks and parts of the chromosomal origin and terminus appear to be bound to surface layers of bacterial cells. Although no clear indication of the functional significance of these associations is available, it seems likely that they are important in growth and division of bacteria. The replication forks may interact with energy-generating machinery in the cell membrane more efficiently when they are membrane bound, whereas the more specific association between the membrane and the origin or terminus may be concerned with chromosome segregation. It is widely believed that surface growth mediates nuclear division and that for this process the chromosome needs to be anchored to the junction of old and new cell surfaces (for a review, see reference 18). Proteins that are important in the maintenance of chromosome supercoiling may also be membrane bound (2).

The techniques used in early studies of *Bacillus subtilis* (i.e., hydrodynamic sheer to break the chromosome and genetic transformation to assay individual genes) generated membrane fractions with only modest enrichment for specific genetic markers (for a review, see reference 30). Furthermore, only a small proportion of each marker was membrane bound. More recently, the nucleoid of *B. subtilis* was dissected with restriction enzymes to facilitate isolation of precisely defined chromosomal fragments attached to particulate structures by a method which was minimally destructive to the cell membrane (19, 20). After gentle hypotonic stress, protoplasts of *B. subtilis* were treated with restriction enzymes and fractionated on sucrose gradients. In contrast to the majority of classical observations, specific DNA sequences were not found with the major plasma membrane fraction but were bound to a small particulate structure that sedimented at a higher speed. The DNA in this fraction was used to identify molecular clones containing sequences of *B. subtilis* DNA that were specifically located in the fraction. We believe that these are the core of the membrane-bound regions seen in the classical experiments.

In this study, a plasmid which cannot replicate in *B. subtilis* but which can integrate into the chromosome through a region of homology was used to determine the genetic locus of a major particle-bound DNA fragment, as suggested by Haldenwang et al. (6) and other workers (3, 4). In both *B. subtilis* and *Escherichia coli* circular plasmids integrate into the chromosome by a homologous recombination process that results in the presence of the entire plasmid adjacent to the homologous region (6, 15, 16, 26). In some cases tandem repetition of the inserted sequence occurs (5, 23, 36, 37). By testing whether copies of the region are also particle bound, it should be possible to determine whether the capacity to bind the chromosome to the membrane is limited to the normal gene dose.

MATERIALS AND METHODS

Bacteria and growth conditions. Studies of membrane attachment were performed in either a low-sulfate medium (20) or in L-broth containing 0.4% glucose. The strains used included strains 168*trpC2*, MS159 (Table 1), and MS240 (see below). The strains of *E. coli* used were a *dam*$^-$ strain (donated by F. Grosveld) and strain DH1 (7) (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44*).

Plasmid construction. The membrane-bound region of φ529 (20) lies on a 5.2-kilobase (kb) *Hin*dIII-*Bgl*II fragment which was cloned into pJAB1 as shown in Fig. 1. pJAB1 is a derivative of pBR322, which was constructed (in collaboration with J. A. Branigan) from strain JH101 (3) and pTR262 (17) recombined at the *Ava*I and *Pst*I sites to give the chloramphenicol resistance marker from strain JH101 and the tetracycline resistance (*tet*) of pTR262, which is under the control of λ repressor. Cloning into the *Hin*dIII site or *Bcl*I site of the repressor binding sequence allows expression of the *tet* gene in *E. coli*. The plasmid is restricted by *Bcl*I only when it is grown in a *dam*$^-$ strain. pJAB1 cut with *Bcl*I and *Hin*dIII was used to clone the membrane-bound region of φ529. The ligation mixture was used to transform *E. coli* DH1 for tetracycline resistance (20 μg/ml). pMS31 had no unusual effects on cell aggregation (22) or on cell growth of strain DH1. Plasmids prepared from this strain contained principally supercoiled and relaxed circle components. Multimers were an insignificant proportion of the plasmid preparation.

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TABLE 1. Three- or four-factor transduction crosses to determine the order of *cam-1* and *cam-2* relative to *purA* and *guaA*

Cross	Donor strain[a]	Recipient strain[a]	Selected marker	Unselected donor markers		No. of recombinants in the following classes:[b]					% Contransduction of *pur* with *cam*
				A	B	11	10	01	00	Total	
1	MS159	MS149	*cam-1*	*purA*$^+$	*guaA*$^-$	57	100	31	12	200	78
2	MS203	SL670	*cam-1*	*purA*$^-$	*spo0J*	50	92	2	4	148	97
				purA$^-$	*gua*$^-$	33	109	2	4		
				guaA$^-$	*spo0J*	33	2	19	94		
3	MS164	SL670	*cam-2*	*purA*$^-$	*spo0J*	121	121	43	48	341	70

[a] The relevant genotypes of the strains used were as follows: strain MS159, *guaA1 cam-1* (constructed by transformation of pMS31 into strain B4 [*guaA*$^-$ *trpC2*], which was obtained from K. Bott); strain MS149, *purA16*$^-$ *guaA1*$^+$ (17); strain MS203, *guaA1 purA16 spo0J*$^+$ *cam-1* (constructed by transduction of *cam-1* into strain SL670); strain MS164, *cam-2 purA16*$^-$ (constructed by transduction of *cam-2* [see text]); strain SL670, *spo0J* 87.1 (21).

[b] Donor and recipient markers are denoted by 1 and 0, respectively. The first and second digits refer to markers A and B, respectively.

DNA purification, preparation of phage and plasmids, agarose gel electrophoresis, blot hybridization and nick translation. The methods used for DNA purification, preparation of phage and plasmids, agarose gel electrophoresis, blot hybridization, and nick translation have been described previously (20). Plasmids were prepared by the method of Ish-Horowicz and Burke (11).

Genetic methods. Plasmid pMS31 was transformed into competent cultures (27) of *B. subtilis*, and transformants were plated onto L-agar containing glucose (4 mg/ml) and chloramphenicol (5 μg/ml) and incubated at 45°C. Transformants were obtained at a low frequency. Once established, the chloramphenicol marker could be transferred by transformation or transduction at a reasonable frequency to other strains. The following two independent isolates were used for mapping experiments: strains MS159 (*cam-1*) and MS158 (*cam-2*), containing pMS31 in strains B4 and MS149 (19), respectively. *cam-2* was transferred to strain 1A160 (*Bacillus* Genetic Stock Center, Columbus, Ohio) by transformation to give strain MS164 (Table 1). The genomes of these strains were mapped by using three- or four-factor transduction crosses and generalized transducing phage AR9 (14). Stocks of phage AR9 were prepared by using *Bacillus pumilus*. Derivatives of strain MS159 able to grow in the presence of 120 μg of chloramphenicol per ml were selected on L-agar plates. One of these (strain MS240) grew at wild-type growth rates in L-broth containing 100 μg of chloramphenicol per ml or in low-sulfate minimal medium containing 20 μg of chloramphenicol per ml.

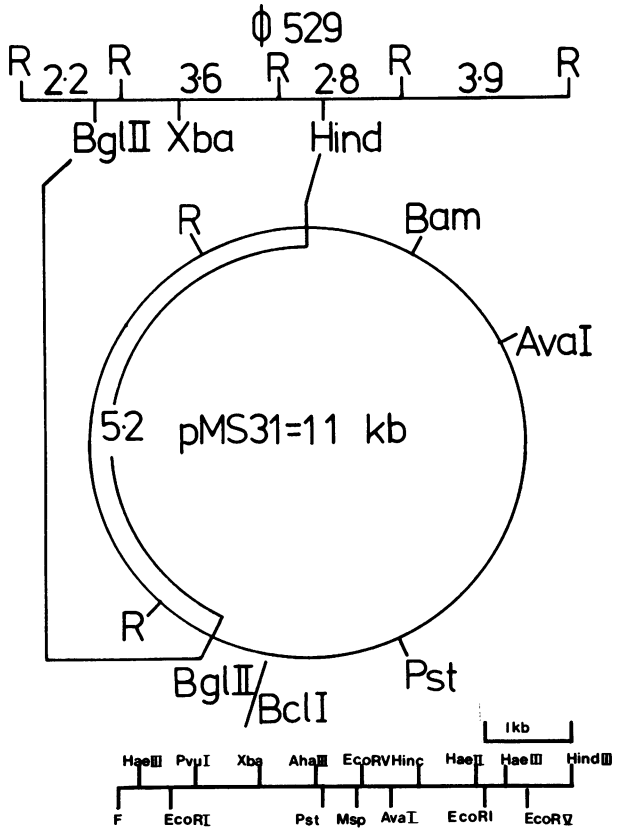

FIG. 1. Origin and structure of pMS31. The 5.2-kb fragment from ϕ529 (flanked by *Hin*dIII and *Bgl*II sites) was ligated into pJAB1 cut at the *Hin*dIII and *Bcl*I sites. The double line indicates the insertion, and the single line indicates the plasmid. The linear map shows the distribution of restriction sites in the 5.2-kb insertion. F, *Bcl*I-*Bgl*II fusion.

Radioactive labeling. DNA was labeled by adding [*methyl*-^{3}H]thymidine (specific activity, 77 Ci/mmol) to a growing culture 1.5 generations before the bacteria were harvested. Proteins were steady-state labeled with $^{35}SO_4$ (specific activity, 10 Ci/mol) in media in which the final sulfate concentration was 2×10^{-4} M.

Preparation of particle-bound DNA. Lysates of *B. subtilis* 168*trpC2*, MS159, or MS240 were treated with *Hin*dIII for 60 to 90 min to give complete digestion after gentle hypotonic lysis (20). The lysates were centrifuged at 10,000 rpm for 10 min to sediment the major membrane fraction. This fraction contained only a small amount of nonspecific DNA and was usually discarded. EDTA (0.05 M) was added to the supernatant, which was layered onto a sucrose gradient (45 ml; 10 to 40% [wt/vol] sucrose on 5 ml of 76% [wt/vol] sucrose in TEN [0.1 M Tris, pH 8.0, 1 mM EDTA, 0.1 M NaCl]). The gradients were centrifuged at 15,000 rpm for 15 h and unpacked from below by using a peristaltic pump. To recover DNA from gradient fractions, samples were incubated with 1% (wt/vol) Sarkosyl at 45°C and then precipitated with 2 volumes of ethanol in the presence of 0.3 M potassium acetate at −20°C for 16 h. Precipitates were dissolved in 0.1× TEN and were purified with phenol (20).

For certain experiments (see Fig. 6), 0.1 mM *p*-hydroxymercuribenzoate was added to the lysis medium (20). This appeared to increase the stability of the particle fraction.

RESULTS

Restriction map of pMS31. The distribution of restriction sites within the *B. subtilis* DNA present in pMS31 is illustrated in Fig. 1. No sites for the following enzymes were found in the insertion: *Bam*HI, *Sal*I, *Sph*I, *Ava*II, *Bgl*II, *Hha*I, *Sst*I, *Pvu*I, *Apa*I, *Hpa*I, *Sma*I, *Xho*I, *Nde*, and *Nco*.

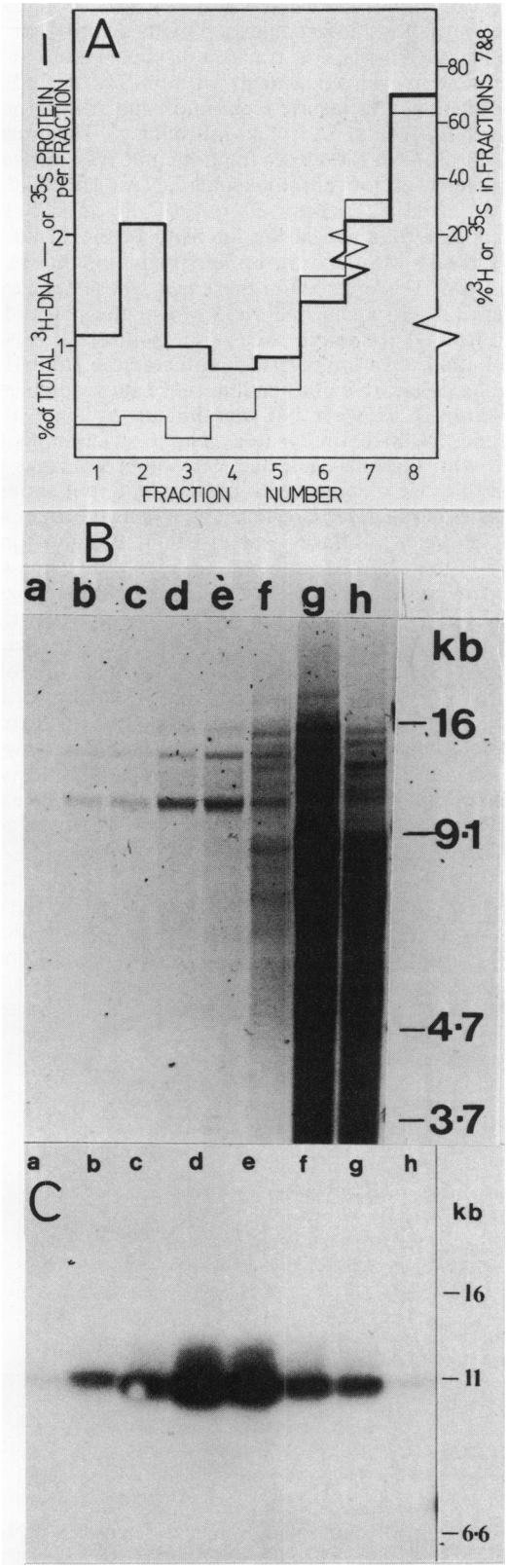

FIG. 2. Sucrose gradient fractionation of *Hin*dIII-digested lysates of *B. subtilis* 168*trpC2*. (A) Distribution of ^{3}H-labeled DNA (light line) and ^{35}S-labeled protein (heavy line) as percentages of totals. Fraction 1 is the bottom of the gradient. (B) Agarose (0.6%) gel electrophoresis of DNA in gradient fractions. Tracks a through h contained the DNAs precipitated from 0.5-ml portions of fractions 1 through 8, respectively (total volume of fractions, 6 ml). (C) Southern hybridization in which pMS31 was used to probe the DNAs shown in Fig. 2B.

Genetic mapping of the pMS31 region. Once introduced into *recE*$^+$ strains of *B. subtilis*, the chloramphenicol resistance gene (*cam*) of pMS31 could be transferred to other strains by transduction or transformation at a reasonably high frequency. We attempted to establish that *cam* was not present as a free plasmid. Samples of lysate were electrophoresed on agarose, transferred to nitrocellulose, and probed with nick-translated pMS31. No free plasmid was observed (data not shown). Freshly isolated transformants were generally sensitive to 20 μg of chloramphenicol per ml, but after storage strains resistant to 20 μg or more of chloramphenicol per ml emerged without selection. These strains contained amplified pMS31 regions.

In all of the transduction crosses shown in Table 1 chloramphenicol was used as the selective marker. Low frequencies of transduction were obtained when chloramphenicol was used unselectively, as noted by other workers (26), possibly as a result of recombination between the homologous regions that flank the plasmid. Transduction crosses were performed with two *cam* isolates obtained by transformation of pMS31 into different recipients (strains B4 and MS149) (see above). Both markers mapped close to *purA16* (70 to 96% cotransducible). The accepted order of the markers in the region (10, 25) is *purA-spo0J-guaA*. In a four-factor cross (Table 1, cross 2), *cam-1* mapped to the left of *spo0J*, but as there was not a clear-cut least-frequent recombinant class, it could not be placed with certainty relative to *purA*. Transformation data (not shown) indicated that *purA* or *spo0J* could be cotransformed with *cam* at a low frequency (about 10%) but not with all three markers. However, the level of congression with unlinked markers, such as *hisA*, was substantial (4%). Therefore, *cam* was placed tentatively between *purA* and *spo0J*.

Cell fractionation studies of the pMS31 region in strain 168*trpC2*. When all of the available sites in lysates of *B. subtilis* were digested with *Hin*dIII, 99% of the chromosomal DNA were dissociated from the membrane fraction that was sedimentable by low-speed centrifugation (10,000 rpm, 5 min) (20). The DNA which remained with the plasma membrane was nonspecific and was probably present in unlysed cells. When the supernatant was fractionated on a sucrose gradient, a small proportion of the DNA and protein sedimented substantially faster than the bulk of the DNA and protein in the cell (Fig. 2A). The DNA in this fraction was present in a small number of restriction fragments, the most prominant of which was 11 kb long (Fig. 2B). When probed with pMS31, this major band hybridized with pMS31 (Fig. 2C); it was present in the highest concentrations in fractions 4 and 5 but was detectable to some extent in the bulk DNA fractions.

Effect of tandem repetitions of the pMS31 region on association with the particle fraction. Integration of plasmids into chromosomes by homologous recombination probably results in duplication of the region of homology (6) and sometimes in tandem repetition of the region (36, 37). The expected behavior of the insertional plasmid during integration into the *B. subtilis* chromosome is shown in Fig. 3. In addition to the normal wild-type 11-kb *Hin*dIII fragment that is homologous to pMS31 (20), there should be an 11.0-kb insertion composed of the cloned 5.2-kb region and pJAB1

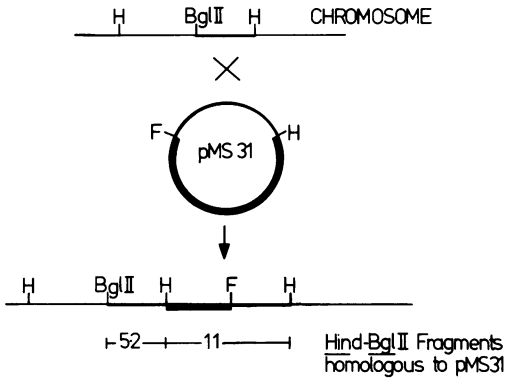

FIG. 3. Mechanism of insertion of pMS31 into the chromosome of *B. subtilis*. F, Position of the *BclI-BglII* fusion; H, *Hin*dIII site. The heavy line indicates the vector sequence. The solid bar indicates the region of homology.

(5.8 kb). These components could not be distinguished without a second digestion. The most convenient method was to use the *Bgl*II site which is present in the wild-type sequence but which was lost from the pMS31 sequence as a result of the *BclI-Bgl*II fusion (Fig. 3, site F).

To confirm this structure, 2 μg of strain MS159 DNA and wild-type strain 168*trpC2* DNA digested to completion with *Hin*dIII or *Hin*dIII plus *Bgl*II were electrophoresed on agarose (0.6%) and probed with pMS31 (Fig. 4), using blot hybridization. Fragments that were about 11.0 and 5.2 kb long and hybridized with pMS31 were obtained from strain 168*trpC2* in *Hin*dIII digests and *Hin*dIII-*Bgl*II double digests, respectively (Fig. 4, tracks g and h). In *Hin*dIII digests of strain MS159, a considerably more intense spot at about 11 kb was also observed (track e). After double digestion with *Bgl*II and *Hin*dIII, there remained a strong band at 11 kb and a weak band at 5.2 (less than 5% of the intensity of the 11-kb band) (track f). The *Bgl*II-resistant 11.0-kb fragment had the properties expected of pMS31 inserted into the chromosome (Fig. 3). The difference in the intensities of the 5.2- and 11-kb fragments suggested that the inserted sequence was amplified to at least 20 copies per wild-type sequence, as reported elsewhere (5, 36, 37). The amplified sequence was clearly evident in ethidium-stained gels containing *Hin*dIII-digested strain MS159 DNA when they were compared with strain 168*trpC2* gels (Fig. 4). The remainder of the pattern clearly indicated that the DNA was completely digested. The culture used for this experiment was grown in medium containing 5 μg of chloramphenicol per ml from a single colony stored on L-agar plates containing the same concentration of the drug. However, it was resistant to at least 20 μg of chloramphenicol per ml when it was subsequently tested. We believe that this resulted from a spontaneous amplification event, as initial isolates from transformation were sensitive to concentrations of chloramphenicol greater than 10 μg/ml.

We wanted to know whether the repeated sequences in chloramphenicol-resistant strains such as strain MS159 were particle bound. To study this further, we obtained a derivative of strain MS159 that was resistant to 120 μg of chloramphenicol per ml (strain MS240). Lysates of strain MS240 were treated with *Hin*dIII and fractionated on a sucrose gradient as described above. The DNA from each fraction was purified by using phenol and then further digested with *Hin*dIII alone or with *Hin*dIII plus *Bgl*II. Figure 5 shows the pattern of restriction fragments seen by ethidium fluoresence and blot hybridization when pMS31 was used as the probe. A heavy ethidium-stained band at 11 kb was prominant in the lower fractions after digestion with *Hin*dIII or with *Hin*dIII plus *Bgl*II (Fig. 5A, tracks a through e and i through m). This band hybridized strongly with pMS31 (Fig. 5B) and was presumably the inserted plasmid, and it was not digested with *Bgl*II (Fig. 5A, tracks i through p). This fragment was also present in the upper fractions but was masked by the remainder of the chromosomal DNA. The wild-type fragment (5.2 kb) was seen after *Bgl*II digestion but was present at less than 5% of the intensity of the 11-kb band (Fig. 5B, tracks i through p). Larger fragments that hybridized with pMS31 were seen in these gels; these appeared to be dimers (22 kb) and trimers (33 kb) of the plasmid that resisted *Hin*dIII treatment. We assume that the repeated particle-bound structure partially protected the *Hin*dIII site. After really exhaustive purification, these sites were susceptible to *Hin*dIII. However, it was difficult to achieve such purification routinely in lysates. The restriction patterns obtained with this material, and with other enzymes were identical to those obtained with pMS31 (data not shown).

Properties of the pMS31 region. The effects of salt concentration, detergent, RNase, and ethidium bromide on the structure of the pMS31 region were explored by using strain MS240 grown in the presence of 100 μg of chloramphenicol per ml. Lysates prepared as described above were treated with *Hin*dIII. After removal of the low-speed pellet, the supernatant was divided into five parts, to which the following additions or treatments were given: (i) 1 M NaCl; (ii) 1% Sarkosyl (45°C for 15 min); (iii) 10 μg of heat-treated pancreatic RNase per ml (37°C) plus 10 mM magnesium sulfate; (iv) 10 μg of ethidium bromide per ml; and (v) no addition. EDTA was added to all of the preparations except the RNase preparation before treatment; EDTA was added

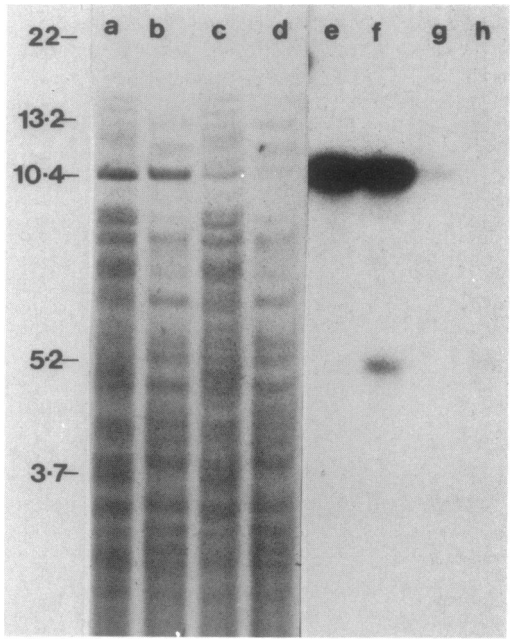

FIG. 4. Southern hybridization of total chromosomal DNAs of strains MS159 and 168*trpC2*. Chromosomal DNAs (2 μg) from strains MS159 (tracks a, b, e, and f) and 168*trpC2* (tracks c, d, g, and h) were digested with *Hin*dIII (tracks a, c, e, and g) or *Hin*dIII plus *Bgl*II (tracks b, d, f, and h) and electrophoresed on 0.6% agarose. Tracks e through h show blot hybridization of tracks a through d, using pMS31 as a probe. Values are sizes (in kilobases).

to the RNase preparation after RNase treatment. Samples were fractionated by ultracentrifugation (Fig. 6). DNA was recovered from each fraction, separated on agarose gels (as for the experiment shown in Fig. 5), and analyzed for homology with pMS31 by using blot hybridization. Figure 6 shows that the pMS31 region (11 kb) was found in the lower gradient fractions (as in Fig. 5) in the control (no addition) and after RNase or ethidium bromide treatment, whereas sodium chloride and Sarkosyl caused this material to unfold and sediment slowly with the bulk of the chromosomal DNA.

The control (Fig. 6, tracks E1 through E7) showed that very little DNA with homology for pMS31 was present in the upper fractions, where the bulk of the DNA sedimented compared with the data shown in Fig. 5. This resulted from an improvement in the lysis conditions (the sulfydral reagent *p*-hydroxychloromercuribenzoate was included in the lysis medium) (see above). We believe that this inhibited an enzyme which was responsible for the instability of the particle fraction.

DISCUSSION

The DNA sequence cloned in pMS31 hybridized with the major 11-kb particle-bound DNA fragment observed after lysates were treated with *Hin*dIII. As it mapped close to *purA*, this fragment is likely to be the critical part of the particle-bound region widely used in previous studies (30). The variability in cotransduction between *purA* and *cam* (70 to 96%) was probably caused by variable amplification of the inserted region during subculture of chloramphenicol-resistant strains. An amplification by tandem repetition would tend to reduce linkage between *cam* and *purA*, the map distance is likely to be an overestimate (27), but is unlikely to be greater than 20 kb (10).

It is now evident that the *purA* membrane attachment site is clearly separate from the chromosomal origin. In a restriction map of the origin region obtained by labeling germinating spores, the origin was placed in a 5.4-kb *Bam*HI fragment contiguous with a 13.1-kb *Bam*HI fragment that encodes the *guaA* gene (21, 22). The 5.4-kb fragment also overlaps a 6-kb *Eco*RI fragment to the left containing the *guaA* gene (13). Both *guaA* and *gyrA* are substantial distances from *purA*, as determined by transduction (25). Previous studies of membrane attachment of DNA in the origin region have indicated that *purA* and ts8132 (closely linked to *guaA*) are attached to the membrane independently of each other (8, 33). No similarity between the previously published restriction maps of the origin and of the pMS31 region have been noted.

The previously described (30) *purA*-enriched particle preparations varied considerably in purity and properties. They ranged from large, relatively impure, salt-insensitive membrane fractions to small, more highly purified, salt-sensitive structures, such as the S-complex (34). The latter contained well-defined restriction fragments (total length, about 50 kb), including a large *Hin*dIII fragment that may correspond to the 11-kb *Hin*dIII fragment of the chromosome to which pMS31 hybridizes.

Like the S complex (34), the amplified particle-bound pMS31 region found in strain MS240 (Fig. 6) unfolds in the presence of high salt concentrations and sediments together with the free DNA fraction. Treatment with detergents has a similar effect (Fig. 6), as noted in the wild-type previously (20). The S complex was also dissociated in vitro in the presence of ethidium bromide (35). However, in our hands, the amplified pMS31 particle fraction was not affected by ethidium bromide. RNase treatment also had no effect on the particle.

The data of Winston and Sueoka (28, 29) suggest that an active *dnaB* gene product (initiation protein) is required both for the formation of the S complex and for the salt-insensitive interaction with the cell membrane. This appears conceptually complicated, as the origin which is presumably the main substrate for *dnaB* and the S complex (or pMS31 region) may be more than 70 kb apart (10). However, this may be clarified when similar studies are performed with defined sequences. As all previously described preparations of particle-bound DNA were isolated after hydrodynamic sheering of lysates, it is not surprising that they contained heterogeneous populations of DNA molecules. The use of restriction enzymes to dissect out a particle-bound DNA, as described above, is experimentally simpler and defines more

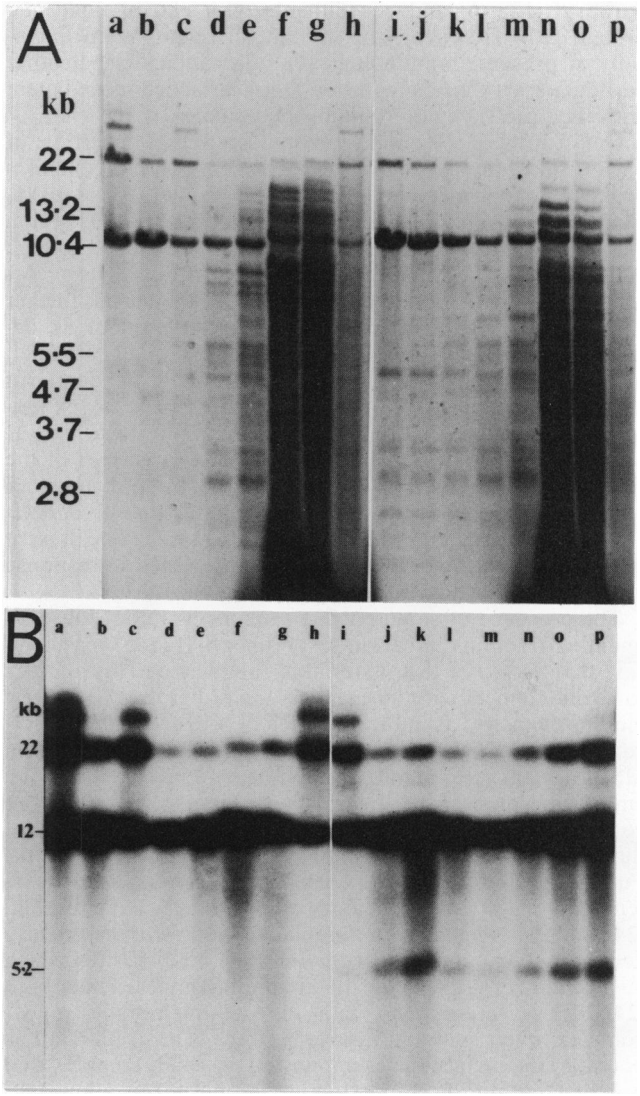

FIG. 5. Sucrose gradient fractionation of a *Hin*dIII-digested lysate of *B. subtilis* MS240. (A) Agarose (0.4%) gel electrophoresis of DNA in a sucrose gradient prepared as described in the legend to Fig. 2. Tracks a through h contained DNAs from fractions 1 through 8 redigested with *Hin*dIII, and tracks i through p contained the same material digested with *Hin*dIII plus *Bgl*II. (B) Southern hybridization in which pMS31 was used to probe the DNA shown in Fig. 5A.

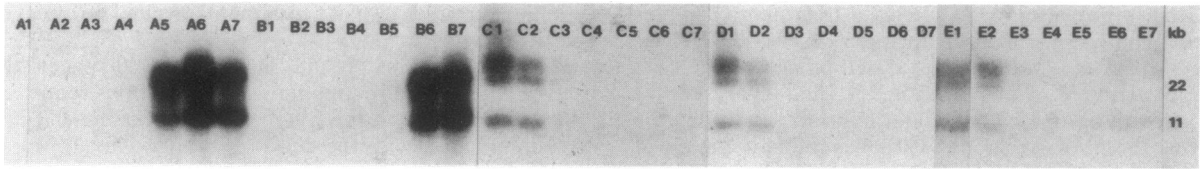

FIG. 6. Properties of particle fraction DNA. Samples (1 ml) of a *Hin*dIII-digested lysate of strain MS240 (after appropriate EDTA treatment and low-speed centrifugation [see text]) were treated with NaCl (tracks A1 through A7), 1% (wt/vol) sodium sarcosinate at 35°C for 15 min (tracks B1 through B7), 10 μg of heat-treated RNase per ml for 15 min at 35°C (tracks C1 through C7), or 10 μg of ethidium bromide per ml (tracks D1 through D7) or were left untreated (tracks E1 through E7). The samples were then fractionated on 10 to 30% sucrose gradients in 0.5× TEN by centrifugation in a Beckman type SW40 rotor at 35°C for 2 h. Tracks A1, B1, C1, D1, and E1 contained the pellet fraction, and tracks A2 through A7, B2 through B7, C2 through C7, D2 through D7, and E2 through E7 contained fractions 1 through 6 (2 ml) from the sucrose gradient (fraction 6 was the top fraction. Southern hybridization was performed on samples of these fractions by using pMS31 as a probe (see Fig. 2).

precisely the sequences involved. We presume that the fast-sedimenting structure containing the pMS31 sequence is of membrane origin, as it is sensitive to the detergent Sarkosyl (Fig. 6) and cosediments with phospholipid and protein (20). However, this is difficult to prove unequivocally at present. Immunological studies indicate that other particulate structures without DNA attached cosediment with the pMS31 region (unpublished data).

Our Southern blot analysis indicated that insertion of pMS31 into the chromosome occurred by a mechanism analagous to the Campbell model, as in many other cases (6, 16, 26). However a 20-fold amplification of the pMS31 region was found in a strain which had not been exposed to high concentrations of chloramphenicol (i.e., strain MS159). After selection with 120 μg of chloramphenicol per ml, further amplification of the pMS31 region was obtained, as described previously for *B. subtilis* (36, 37); B. Niaudet, personal communication) and *E. coli* (5). The exact degree of amplification appears to vary from experiment to experiment. More surprising than the amplification of the pMS31 region, however, was the association of a large part of the repeated sequences with the particulate fraction together with the wild-type sequence. This suggests that *B. subtilis* has an excess capacity to bind the region that may be present during normal growth or that may be produced in response to the increased dose of the pMS31 region.

The presence of some pMS31 sequences in the slow-sedimenting fractions in certain experiments (Fig. 5) may indicate that not all of this material is complexed. This appears to be due to instability during isolation rather than to limiting binding capacity; as in the presence of *p*-hydroxychloromercuribenzoate (Fig. 6) almost all of the pMS31 region in strain MS240 is particle bound. The observed enrichment of the amplified region in the lower part of the gradient is also of considerable practical value, as greatly increased yields of the complex can now be obtained for biochemical work. Furthermore, it provides a considerably simpler model system than one involving an autonomously replicating plasmid (24, 28, 29). Plasmids that replicate in *B. subtilis* clearly contain one or more specific membrane binding sites which may be concerned with plasmid initiation or partition or both (24). These interactions with the cell membrane appear complex even without inserted DNA. Thus, pUB110 is bound to the membrane as a high-salt-resistant complex that is dependent on *dnaB* function in vivo but forms a low-salt-resistant *dnaB*-independent complex in vitro (24).

The pMS31 region is not the only membrane-bound part of the chromosome. The restriction patterns of membrane-bound DNA suggest that there may be up to 12 separate membrane-bound fragments, some of which may be contiguous on the chromosome (20). A strong membrane attachment in the *ilvA-ilvD* region has been described previously (19), and there appears to be another very close to the origin at *guaA* (1) and ts8132 (8).

Recent work on *E. coli* indicates that there is a major interaction between several points in the origin region and the outer membrane (9, 12, 31). Elevated levels of the proteins mediating this interaction have been found in strains that are diploid for the region (32).

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