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# Identification of a Specific Membrane-Particle-Associated DNA Sequence in *Bacillus subtilis*

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**After the *Bacillus subtilis* nucleoid was dissected with restriction endonucleases, a specific DNA sequence from the *purA* region was isolated in a particulate form that probably originated from the cell membrane. Precise definition of the binding region within this sequence was achieved by a novel procedure based on a previously reported observation that additional copies of the binding region, introduced into the chromosome using an integrative plasmid, were also predominantly particle bound. Subsections of the original plasmid insertion were cloned into the integrative plasmid and introduced into *B. subtilis*, in which they became tandemly reiterated under appropriate selective conditions. *Hae*III sites in the vector, flanking each insertion, were used to excise the latter for subsequent tests of particle association. Examination of 10 strains containing subsections of the original 5.2-kilobase-pair region showed that the binding region was confined to 283 base pairs. This was confirmed by dissection in vitro of a larger, isolated, particle-bound sequence. The nucleotide sequence of a 1,300-base-pair region that contained this site was determined. The entire region had a notably high A+T content and was deficient in open reading frames for transcription.**

The existence of a membrane-bound DNA sequence close to the *purA* marker in the *Bacillus subtilis* chromosome has been suspected for many years, based on the observed enrichment of *purA* transforming activity relative to midchromosomal markers (15). Initially, this sequence was believed to be part of the origin of replication and, therefore, to be intimately involved with chromosome initiation and segregation, but it is now clear that the origin lies at least 50 kilobase-pairs (kb) to the right of *purA*, close to *guaA* and *gyrA* (5, 6, 10). Subsequent studies have led to the identification of a small particulate fraction (the S-complex) that contains a substantial proportion of the *purA* transforming activity and DNA in a rosettelike structure. Although this particle was isolated from randomly sheered lysates, it contains a core sequence with a defined restriction map of about 50 kb (16).

A structure which may be related to the S-complex has also been isolated from lysates of *Bacillus subtilis* after digestion with restriction enzymes (9, 10, 11). Such treatments released essentially all of the DNA from the major plasma-membrane fraction, but a particulate structure containing a small number of specific sequences could be sedimented at high speed from the released DNA material. These small structures could be disrupted by high concentrations of salt, proteases, and detergents (10). Molecular clones containing the DNA portion of these structures were obtained and were used to show that one particle-bound sequence was confined to a 5.2-kb *Hin*dIII-*Bcl* fragment. This maps close to *purA* and is part of an 11-kb *Hin*dIII fragment (10), which may correspond to a fragment of similar size in the S-complex (16). Difficulties encountered in further attempts to dissect the particle-binding site within the 5.2-kb region with other enzymes led us to devise an alternative strategy for delimiting the binding region. This exploited the observation that nonreplicative plasmids containing the 5.2-kb membrane-binding sequence integrated into the *B. subtilis* genome by homologous recombination and became tandemly reiterated under appropriate selection conditions (10). A substantial proportion of the additional copies were also particle bound and could be excised at restriction enzyme sites in the vector sequences that flank the insertion. A variety of subclones of the 5.2-kb sequence were therefore constructed and introduced into *B. subtilis* in the tandemly reiterated form. These strains were examined to see whether the reiterated DNA was in a particle-bound form.

In this communication we show that the binding region was confined to 283 base pairs within the 5.2-kb region. The nucleotide sequence of a 1,334-base-pair region that included this site was determined.

## MATERIALS AND METHODS

**Bacteria and growth conditions.** The strains used (Table 1) were all derivatives of *B. subtilis* CU1427 (kindly supplied by S. A. Zahler, Cornell University) *ilvA ilvD trpC2 att* (SPβ). All *B. subtilis* strains used were grown in L broth containing 0.4% glucose and 100 μg of chloramphenicol $ml^{-1}$.

Plasmids were prepared in *Escherichia coli* DH1 (10).

**Plasmid construction.** Subclones of pMS31 (10) were constructed as shown in Table 1 and Fig. 1, by standard methods for restriction and ligation. The vector used, pJH101, is able to replicate in *E. coli* but not in *B. subtilis* (3, 10).

**DNA purification, preparation of plasmids, agarose gel electrophoresis, blot hybridization, and preparation of radioactive probes.** The methods used for DNA purification, preparation of plasmids, agarose gel electrophoresis, restriction analysis, and blot hybridization have been described previously (10). Radioactive probes were prepared with [$^{32}$P]dCTP by the method of Feinberg and Vogelstein (2).

**Genetic methods.** Newly constructed plasmids, prepared in *E. coli*, were introduced into competent cultures of *B. subtilis* by a standard transformation method (10), plated onto L agar containing glucose (4 mg $ml^{-1}$) and chloramphenicol (5 μg $ml^{-1}$), and incubated at 45°C. Transformants were obtained at low frequency. These arise by integration of the plasmid into the chromosome by homologous recombination (10). Subsequent selection for resistance to high

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TABLE 1. Subclones of pMS31 used to delimit particle binding site

| Plasmid | Size of *B. subtilis* insertion (kb) | *B. subtilis* strain containing reiterated sequence[a] | Construction or source[b] | Size of *Hae*III fragment surrounding insertion (kb)[c] | Membrane association |
|---|---|---|---|---|---|
| pMS31 | 5.2 | MS159 | 11 | 4.0 | + |
| pMS90 | 2.85 | MS241 | *Pst-Hin*dIII fragment into JH101 | 2.1 + 0.6[d] | + |
| pMS91 | 3.6 | MS242 | *Eco*RI fragment into JH101 | 3.69 | + |
| pMS92 | 1.0 | MS246 | *Eco*RI (B)-*Hin*dIII fragment into JH101 | 0.24 + 0.61[d] | − |
| pMS96 | 1.85 | MS267 | *Pst-Eco*RI (B) fragment into JH101 | 2.09 | + |
| pMS98 | 1.6 | MS257 | *Eco*RI (A-B) deletion of pMS31 | $<1 + 0.5 + 0.6$[d] | − |
| pMS105 | 1.45 | MS258 | *Pst-Hinc* fragment into JH101 | 1.68 | + |
| pMS106 | 0.5 | MS274 | *Pst-Eco*RV fragment into JH101 | 0.65 | − |
| pMS120 | 1.3 | MS273 | *Pst-Ava*I fragment into JH101 | 1.48 | + |
| pMS122 | 0.67 | MS275 | *Alu* fragment (C-D) in *Eco*RV site of JH101 | 0.8 | − |
| pMS123 | 0.67 | MS276 | *Alu* fragment (A-B) in M13 (excised with *Pst-Hin*dIII into JH101 | 1.0 | + |

[a] All *B. subtilis* strains constructed in CU1427 (*trpC2 ilvA ilvD att* [SPβ]), supplied by S. A. Zahler.
[b] All constructions in pJH101 (3) at sites shown, unless stated otherwise.
[c] Sum of insertion plus flanking sequences to *Hae*III sited in vector.
[d] Two or three fragments present owing to *Hae*III sites in vector sequences. Similar results were obtained by using *Hin*dIII-digested lysates as described in reference 11.

concentrations of chloramphenicol (100 $\mu$g ml$^{-1}$) led to tandem reiteration of the integrated plasmid.

**Preparation of lysates and isolation of particle fractions.** Bacteria to be lysed were grown to an optical density (OD) at 540 nm ($OD_{540}$) of 2.0 (0.4 mg ml$^{-1}$ [dry weight]). Protoplasts were prepared at 40 OD U ml$^{-1}$ and subsequently lysed by gentle hypotonic stress exactly as described previously (10; final volume, 1 ml). These were incubated at 35°C for 1 h with either *Hae*III or *Bsu*R (10 U per OD U of cells). Lysates were then centrifuged at 10,000 rpm for 10 min to remove unlysed cells and the major part of the plasma-membrane fraction. Small amounts of DNA remained with this fraction. EDTA (0.01 M) was added to the supernatant which was then applied to a 10-ml, 10-to-30% linear sucrose gradient (in 0.05 M Tris hydrochloride [pH 7.5], 0.05 M sodium chloride, 0.05 M EDTA) and centrifuged in Beckman SW40 10-ml tubes at 39,000 rpm for 2 h. DNA was removed from the fractions by ethanol precipitation. (10).

For studies in which partially purified particle fraction DNA was to be treated with other enzymes (see Fig. 2), the particle fraction was pelleted in a Beckman SW40 rotor at 39,000 rpm for 90 min. The pellets were suspended in 0.01 M Tris hydrochloride [pH 7.5]-0.01 M NaCl-0.1 mM EDTA and then digested with other enzymes in the presence of 5 mM $MgCl_2$. The reactions were terminated with EDTA (0.05 M) after 1 h, and the products were fractionated on 10-ml sucrose gradients as described above.

**Analysis of nucleotide sequence.** The nucleotide sequence of pMS120 was obtained by using the dideoxynucleotide method (8) with subclones of pMS120 prepared in phage M13mp18 and -19 (Fig. 3).

## RESULTS

**Construction of subclones of the 5.2-kb membrane-binding sequence and integration into the *B. subtilis* chromosome.** Ten subclones of the 5.2-kb region in pMS31 were prepared in pJH101 as shown in Fig. 1 and Table 1. This plasmid replicates in *E. coli* but not *B. subtilis* (3, 10). When introduced into *B. subtilis* CU1427 by transformation, selecting for resistance to chloramphenicol (5 $\mu$g ml$^{-1}$), the plasmid underwent homologous recombination with the host chromosome by a mechanism analogous to the Campbell model of phage insertion, giving two copies of the inserted region. Each of the plasmids described in Table 1 was stably integrated in this way, and no significant amount of free plasmid was present (data not shown). Subsequent growth of these strains in the presence of higher concentrations of chloramphenicol selected strains that were resistant to 100 $\mu$g ml$^{-1}$ of the antibiotic. These contained multiple copies (about 20) of the inserted plasmid in a tandem array as shown previously for a similar strain (10).

Strain CU1427 was used as a recipient since we have shown that SPβ$^-$ strains have substantially less nuclease activity than SPβ$^+$ strains in lysates, under the conditions used for preparing particle fractions (12).

**Delimitation of the membrane-binding site within the 5.2-kb sequence by using strains containing reiterated subclones of the region.** Many *Hae*III (or *Bsu*R) sites are present in pJH101, whereas most of the subcloned regions (Table 1) lack *Hae*III sites. This enzyme can therefore be used to excise the insertion regions of each plasmid from strains containing reiterated sequences. The procedure used was to digest lysates of each strain (Table 1) as completely as possible with *Hae*III, remove unlysed cells and the bulk of the plasma-membrane fraction with a low-speed centrifugation, and then fractionate the supernatant by density gradient centrifugation. DNA from the gradient fractions was purified and analyzed by blot hybridization with an appropriate radioactively labeled part of the pMS31 insertion. Under the conditions used for the high-speed centrifugation step, the bulk of the DNA remains at the top of the gradient (data not shown; see reference 10), while any particle-bound DNA was found in the lower fractions (Fig. 4). The relatively high sedimentation coefficient of the particle fraction compared with the bulk of the DNA is dependent on association with protein material. Proteases, detergents, and high-salt treatments reduce the sedimentation coefficient of this material to that of the bulk of the DNA (data not shown; see reference 10). Fast sedimenting particles containing sequences homologous to some part of the pMS31 region were found in 7 out of 10 subclones examined (Table 1). The criterion used to classify these strains with respect to membrane association was whether a significant hybridization signal was obtained in the fast-sedimenting region. Attempts to present this data in a more quantitative fashion did not illuminate the problem further. Examples of the behavior of

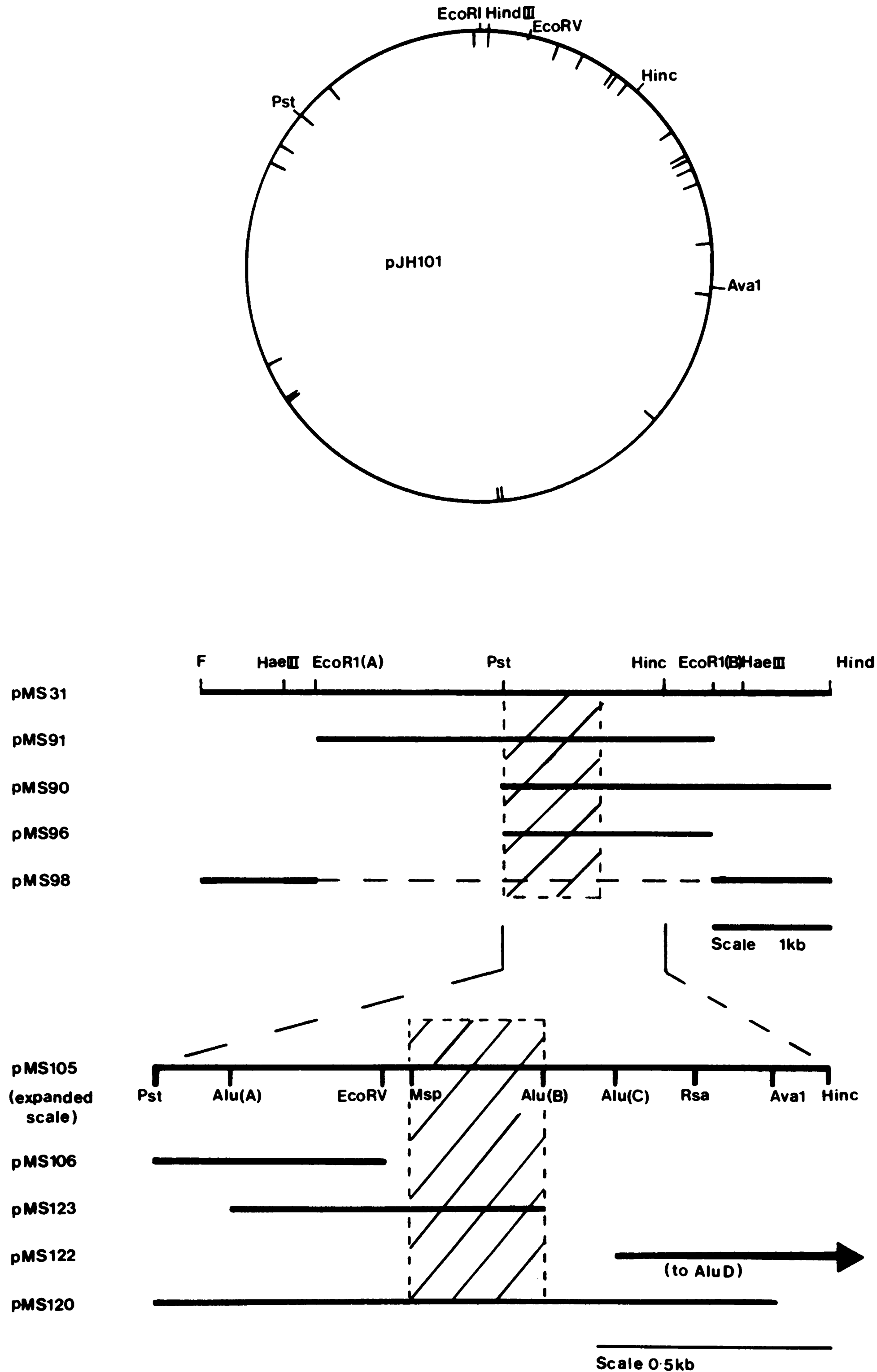


FIG. 1. Origin of subclones of pMS31. Top, pJH101 (3) showing disposition of *Hae*III sites (inside marks) and sites used for cloning (outside marks). Bottom, Linear map of pMS31 and subclones of pMS31, constructed as shown in Table 1. Hatched areas, Particle-binding sites; F, *Bgl*II-*Bcl* fusion.

four of these strains are illustrated in Fig. 4. In strain MS273, the inserted plasmid (pMS120) was particle associated (Fig. 4A). A fast-sedimenting fragment of about 1.4 kb, corresponding in size and hybridization characteristics to the *Hae*III fragment carrying the inserted DNA, was found in the lower part of the gradient. Material of the same size was seen in the upper fractions and was presumably not particle bound or had dissociated from the particle during prepara-

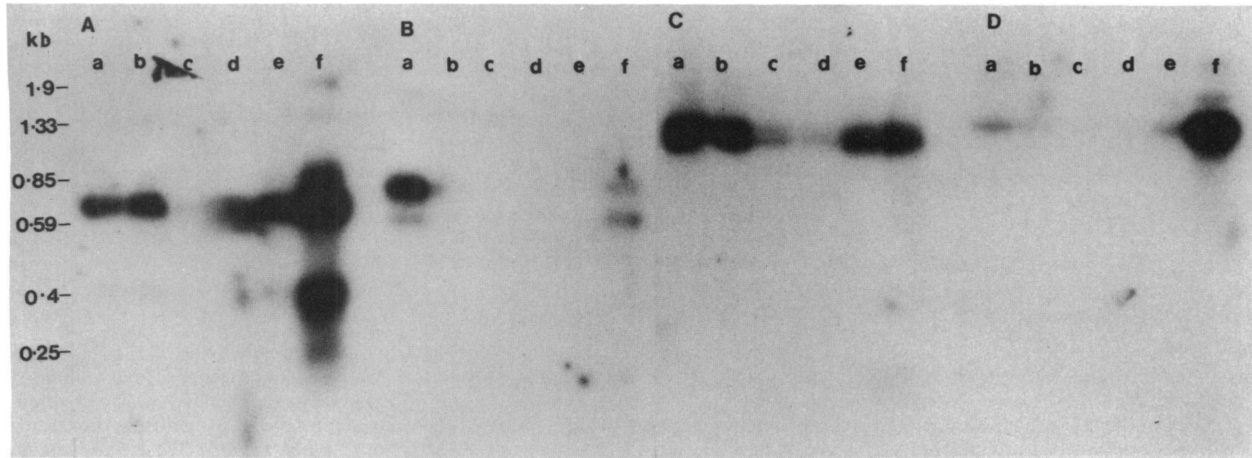


FIG. 2. Mapping of the particle-bound region by using secondary digestions of particle-bound DNA from a strain containing pMS120. Lysates of strains containing tandemly reiterated pMS120 were treated with *Hae*III as described in the legend to Fig. 4 and in the text. After the removal of the low-speed sediment and the addition of EDTA, the lysate was centrifuged, and the pellets were suspended and then digested in the presence of 5 mM $MgCl_2$–*Hae*III alone (D) or 5 mM $MgCl_2$–*Hae*III plus (A) *Alu*, (B) *Msp*, or (C) *Rsa*. The reactions were terminated with EDTA, and the products were fractionated on sucrose gradients as described in the text. After recovery from the gradients, the DNA was analyzed by blot hybridization as described in the text. Tracks a through f show gradient fractions from the bottom to the top, respectively (panel A contains 3 times as much material as the others). The probe used was the entire *Hae*III nonvector fragment of PMS120 (Fig. 4).

tion. Fragments larger than 1.4 kb, which are partially digested sequences, were also seen in the gradient. In strain MS276, the insertion of pMS123 was present as a fast-sedimenting 1-kb sequence. It included the 0.67-kb *Alu*A-*Alu*B sequence (see Table 1).

Strains that did not contain the sequence necessary for association with the particle fraction are shown in Fig. 4B and C (i.e., MS274 and MS275 containing pMS106 and pMS122, respectively). In both strains, the fragments containing the inserted DNA were found at the top of the gradient (0.725 and 0.8 kb, respectively). These strains contained sequences from the right- (pMS122) and left-hand

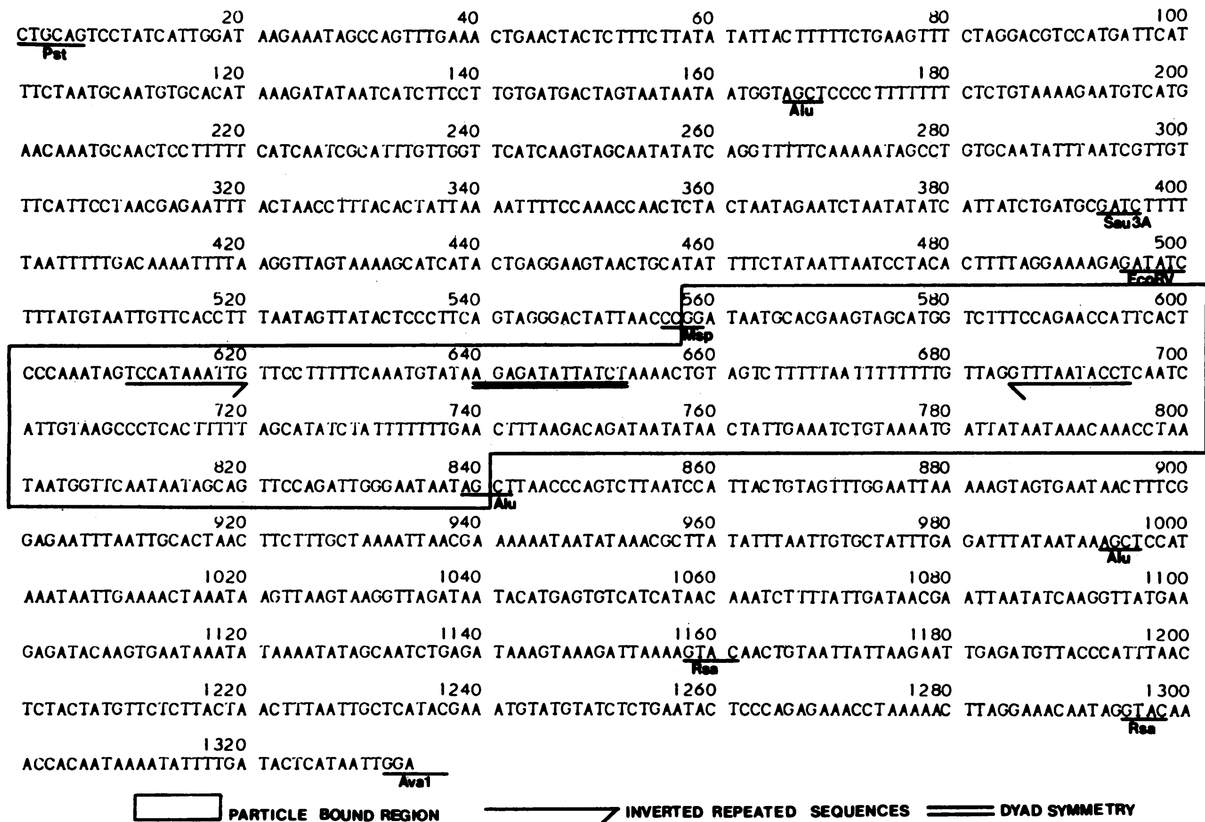


FIG. 3. Nucleotide sequence of *Pst*-*Ava*I fragment from pMS120.

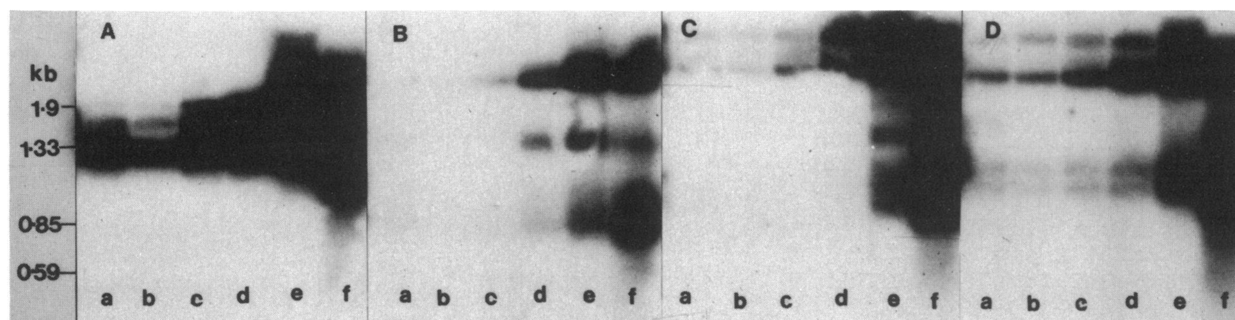


FIG. 4. Mapping of the particle-bound region by using *B. subtilis* strains containing linearly reiterated subclones of pMS31. Lysates of strains containing tandemly reiterated plasmids were prepared by gentle hypotonic stress (see Materials and Methods; final volume, 1 ml), incubated with *Hae*III (100 U per 2 mg [dry weight] of bacteria) and then centrifuged as described in the text. The DNA was recovered from 2-ml samples of the sucrose gradient by ethanol precipitation and was then fractionated by electrophoresis on 2% agarose. After transfer to nitrocellulose the DNA was hybridized with a nick-translated *Hae*III fragment of pMS120 containing *B. subtilis* DNA. The behaviors of strains of *B. subtilis* containing pMS120, 106, 122, and 123 are shown in panels A through D, respectively. Tracks a through f show gradient fractions from the bottom to the top, respectively. Size calibration is in kilobase pairs.

(pMS106) ends of the pMS120 sequence (Fig. 1). It appeared, therefore, that the specificity for particle association lay to the right of *Eco*RV and to the left of *Alu*B (Fig. 1). The behavior of six other strains that were tested (Table 1, Fig. 1) was also consistent with this conclusion (data not shown).

**Delimitation of the particle-binding sequence by secondary digestion of isolated particle-bound DNA.** Further confirmation of the map position of the binding region has been obtained by digesting the isolated particle-bound *Hae*III fragments of MS273 (containing pMS120) with other enzymes (Fig. 2). When digested with *Alu* (Fig. 2A), a 0.675-kb fragment was fast sedimenting (i.e., particle bound), while at the top of the gradient, fragments of 0.4 and 0.25 kb were observed. The 0.675-kb fragment was clearly the *Alu*A-*Alu*B fragment at the center of pMS120, while the 0.4- and 0.25-kb fragments originated from the right of *Alu*C and the left of *Alu*A, respectively. The small *Alu*B-*Alu*C fragment (154 base pairs) could not be detected (Fig. 1). The restriction enzyme *Msp* divided the pMS120 insertion into 0.8- and 0.6-kb right and left portions, respectively (Fig. 1). The former was most enriched in the fast-sedimenting fraction, while the latter was at the top of the gradient (Fig. 2B). After *Rsa* treatment, the left-hand part of the sequence (1.1 kb) was particle bound (Fig. 2C). Secondary digestion of the isolated pMS120 binding region suggests that the sequence conferring particle association was delimited by the *Msp* and *Alu*B sites.

**Nucleotide sequence of the pMS120 insertion.** The entire sequence of pMS120 (Fig. 3) has been determined by the dideoxynucleotide method of DNA sequencing with M13 subclones (8). The origin of these clones and the direction of sequencing are shown in Fig. 5.

## DISCUSSION

The 5.2-kb region of the *B. subtilis* chromosome that maps near *purA* has been identified as a possible membrane-binding sequence (10). Multiple copies of this sequence,

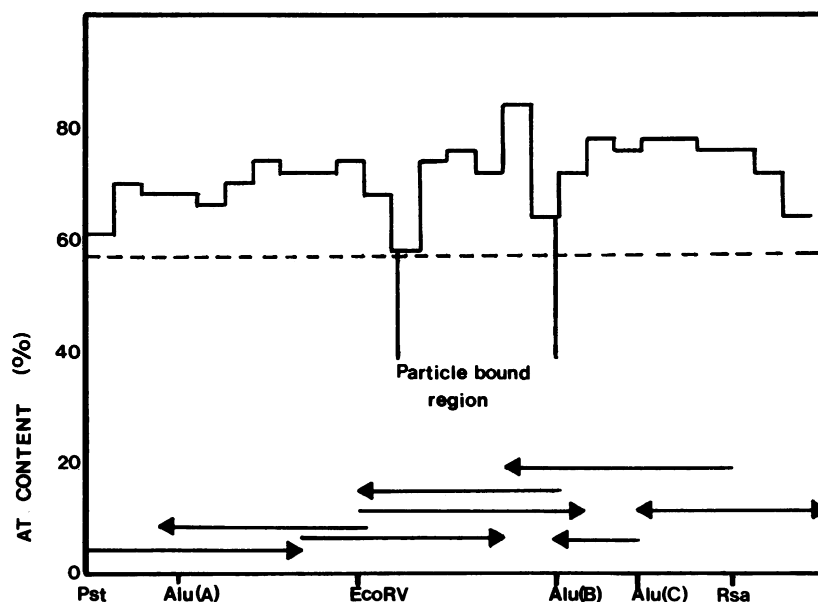


FIG. 5. Distribution of A+T base pairs in *Pst*-*Ava*I fragment from pMS120. The ordinate is a linear restriction map of the *Pst*-*Ava*I fragment. A+T content for 50 base units is shown. Arrows, M13 clones sequenced; -----, average A+T content of *B. subtilis* DNA (57%).

when present in the *B. subtilis* genome, were also bound to particulate structures, thus indicating that these bacteria have excess capacity to bind this region. This fortuitous discovery has enabled us to devise a versatile procedure by which the binding region could be delimited within the 5.2-kb region with a set of subclones of this sequence and one inexpensive restriction endonuclease.

An alternative approach would have been to clone putative binding regions in an autonomously replicating plasmid. This course, however, suffers from a serious difficulty as the replicative apparatus of such plasmids also binds to the membrane (13, 14) and would therefore have led to a complicated interaction. The presence of multiple copies of the binding region in positive strains, containing tandem reiterations, also gave a more conclusive demonstration of particle association than studies with single-sequence strains.

Analysis of strains containing the 10 subclones described above has indicated that the regions to the right of *Eco*RV and to the left of *Alu*B were necessary for membrane association. Confirmation of this conclusion was obtained by secondary digestions of the binding region from MS273 (containing pMS120). The particle-bound pMS120 region, on redigestion with *Msp* after isolation from gradients, gave a bound 0.8-kb fragment. This fragment originated from the right of pMS120 (Fig. 1). Digestion with *Alu* gave a bound 0.675-kb fragment, which was from the middle of pMS120 and surrounded the *Msp* site. The binding region therefore appeared to be present in a 283-base-pair region from *Msp* to *Alu*B (Fig. 1). Similar experiments have been performed with larger insertions (i.e., pMS91 and pMS96) and have given similar results (unpublished observations). Independent support for this conclusion has also been obtained by the isolation of a DNA-binding protein complex, from a particulate fraction of a cell, that associates specifically with this sequence (M. G. Sargent, unpublished observations).

The entire pMS120 insertion was sequenced. The region was notable for its high A+T content (70%) and absence of possible transcriptional reading frames. A pair of short inverted repeats (not quite perfect) and a short sequence of dyad symmetry within the 283-base-pair binding region may have significance in the binding reaction. The A+T content of the binding region was also slightly lower than that of the flanking region (Fig. 5). The high A+T content of the flanking region may provide an explanation of the observation that membrane-binding sites demonstrated in *E. coli* by a different technique are sensitive to endonucleases that attack only single-stranded regions (1). A+T-rich sequences adjacent to these membrane-binding sites may become susceptible to the nuclease as a result of localized breathing of the DNA duplex.

As we suggested above, the cytological significance of small particulate fractions such as the S-complex (16) or the fraction described here is difficult to assess. The most widely favored view is that this fraction is derived from the membrane and represents an attachment point of the chromosome to the membrane. We have suggested previously that cutting of the tightly coiled chromosome releases forces capable of pulling attachment sites out of the membrane (10, 11). We noted that the apparently gentle process of digestion of the chromosome with restriction enzymes released up to 30% of the phospholipid from the plasma-membrane fraction that sediments at low speed (9, 11). Furthermore, the structural domain of the cell membrane to which the chromosome is most likely to be attached (the mesosome) is a notoriously evanescent entity of uncertain biochemical significance (4).

Another possible explanation of the particle-bound DNA complexes which cannot be discounted is that they originate from the core of the nucleoid and may under certain circumstances become trapped in membrane vesicles during isolation (7). The route to solving the problem of the cytological origin of these complexes may be to identify proteins that bind specifically to this sequence and then to determine their intracellular location.

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