Specific in vitro binding of a plasmid to a membrane fraction of Bacillus subtilis

(Staphylococcus aureus plasmids/pSL103 and pUB110/initiation mutants)

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ABSTRACT A model system has been developed to study the in vitro binding of a plasmid to the membrane fraction from Bacillus subtilis. The plasmid DNA molecule used in these studies was pSL103 (8.0 kilobases), a chimeric plasmid consisting of a Staphylococcus aureus plasmid (pUB110, 4.5 kilobases) and a DNA fragment (3.5 kilobases) from Bacillus pumilus carrying trpC+ gene. This plasmid replicates in B. subtilis cells, and its in vivo membrane binding (as well as its replication) is dependent on the product of a DNA initiation gene, dna-I, of B. subtilis. In this paper we demonstrate the in vitro specific binding of exogenous pSL103 to the isolated membrane fraction. This in vitro binding is specific to the origin-containing portion (pUB110) of pSL103. The trpC+-containing portion neither binds to the membrane fraction nor competes with pSL103 for binding to the membrane fraction in vitro. CoE1 plasmid, which does not replicate in B. subtilis, neither binds to the B. subtilis membrane fraction nor competes with pSL103 for binding.

The association of the origin of DNA replication of the bacterial chromosome with the membrane has been reported in both Bacillus subtilis (1–6) and Escherichia coli (7, 8). This association may serve to regulate in some way the initiation of chromosome replication which is the primary control step in the prokaryotic cell cycle (1). We have tested this hypothesis by determining the effect of initiation-defective mutants of B. subtilis on DNA–membrane association (6). In vivo, the membrane association of DNA near the chromosomal origin in B. subtilis is dependent on the dna-I gene product, which is also required for initiation of B. subtilis chromosomal replication at the origin. Membrane association and initiation of the plasmid pSL103 in B. subtilis are also dependent on the dna-I gene product (6). The plasmid pSL103 (8.0 kilobases (kb)) is a chimera between the Staphylococcus aureus plasmid pUB110 (4.5 kb), which provides a replication origin, and a trpC+-containing DNA fragment (3.5 kb) from Bacillus pumilus (9) (see Fig. 2 Inset).

In contrast to the dna-I mutation (10), the dnaB19 mutation (11) affects only the membrane association and initiation of the B. subtilis chromosome and has no effect on the replication or the membrane binding of pSL103 (6, 12). The temperature-sensitive initiation mutants dna-I and dnaB19 carry defects in distinct but closely linked loci, dnaB1 and dnaB11, respectively (13). When purified DNA–membrane complex isolated from dna-I (dnaB1) cells containing pSL103 is incubated in buffer at the nonpermissive temperature, plasmid DNA and DNA near the B. subtilis chromosomal origin are selectively released from the complex (6).

In this paper we describe a system in which pSL103–membrane complex can be formed in vitro from purified components. Thus, pSL103 can specifically bind in vitro to the isolated membrane fraction from B. subtilis. Furthermore, this binding is specific for the origin-carrying half of pSL103–i.e., pUB110.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. B. subtilis 168 trp thy (pSL103) (6), 168 trp thy dna-1 (10), 168 trp thy dna-1 (pSL103) (6), and 168 leuA8 metB5 purA16 (14) were used. Cells were grown in the SPC+ salt medium (15) at 37°C, except that the strain carrying the dna-I mutation was grown at 32°C in medium supplemented with thymine at 5 μg/ml. The cells were grown to 7 × 107 cells per ml and harvested by centrifugation; frozen cell pellets were stored at −70°C.

Radioactive Labeling. Cells were labeled by growing them in the appropriate medium as described above with 3H-thymidine (50 Ci/mmol; 1 Ci = 3.7 × 1010 Bq; Amersham) at 4 μCi/ml, 14C-thymine (50 mCi/mmol; New England Nuclear) at 1 μCi/ml, or 14C-leucine (50 mCi/mmol; New England Nuclear) at 1 μCi/ml. 3H-Labeled plasmid prepared by growing 168 trp thy cells harboring the plasmid with 3H-thymidine as above had a maximal specific activity of 3.4 × 104 cpm/μg. To increase the specific activity of the plasmid, cells were first grown in the presence of neomycin (5 μg/ml) and thymine (3–5 μg/ml). When the culture reached 7 × 106 cells per ml (40 Klett units), [3H]thymidine was added (4 μCi/ml) to the culture flask along with hydroxyurea to a final concentration of 0.2 M. Hydroxyurea specifically inhibits host chromosomal DNA synthesis but allows the plasmid to continue to replicate; it also increases pSL103 copy number to approximately 200 copies per cell (12). After addition of hydroxyurea and [3H]thymidine, the cell culture was allowed to continue growing for 3 hr (12). The cells were then harvested and stored at −70°C until needed. Plasmids were isolated as described below. The specific activity obtained by this procedure was as high as 3 × 106 cpm/μg of plasmid.

Preparation of Membrane Fraction. Frozen cell pellets from 25 ml of culture were thawed and resuspended in a mixture of 0.5 ml of TKE buffer (0.02 M Tris, pH 8.1/0.1 M KCl/1 mM EDTA) (16), 0.1 ml of lysozyme solution (5 mg/ml), and 0.05 ml of 0.1 M 2-mercaptoethanol. After 15 min at 32°C, 0.1 ml of 5% Brij-58 was added. The solution was allowed to stand at room temperature for 2 min and then placed on ice. The sample was sheared 10 times by passing it through an 18-gauge needle. Sucrose gradient centrifugation, fractionation, and radioactivity assays were as described (6). The portions of the gradient containing the membrane fraction were pooled.

Membrane DNA Isolation. Cell pellets from a 1.2-liter culture of B. subtilis cells harboring pSL103 grown to the stationary phase in tryptophan-free medium were resuspended in 2 ml of

Abbreviation: kb, kilobase(s).

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0.5 M sucrose/0.05 M Tris, pH 8.0. After incubation for 30 min at 57°C, 2.5 ml of 0.25 M EDTA (pH 8.0) was added to the cells and the mixture was incubated for 5 min at room temperature. Then, 8 ml of 1% Brij-58/0.4% deoxycholate/0.05 M Tris/0.025 M EDTA, pH 8.0, was added and the cell lysate was incubated for 10 min on ice. The solution was centrifuged for 20 min at 18,000 rpm in a Sorvall SS34 rotor at 10°C. The supernatant was made 0.88 g/ml in CsCl and 750 μg/ml in ethidium bromide and centrifuged in a Beckman 75Ti rotor for 44 hr at 42,000 rpm at 20°C. The plasmid DNA was visualized by UV fluorescence and the band was removed with a syringe and 20-gauge needle.

Ethidium bromide was removed by extraction with n-butyl alcohol and the solution was dialyzed against 4 liters of 0.1 M ammonium carbonate. The plasmid DNA was either concentrated by ethanol precipitation or by lyophilization to dryness. The DNA was then resuspended in the appropriate buffer. For radioactively labeled plasmid DNA, the CsCl/ethidium bromide gradients were divided into 90 fractions, and 0.015 ml was removed from each fraction for assay of radioactivity. The pooled DNA fractions containing plasmid were treated as described above. The purity of the plasmid was examined by agarose gel electrophoresis (17).

In Vitro Binding. The isolated membrane fraction from 35 ml of culture was dialyzed against 1.5 M sodium citrate/15 mM NaCl/1 mM EDTA overnight and concentrated to approximately 1.5 ml against polyethylene glycol 6000 (PEG 6000). The concentrated membrane fraction was incubated at 45°C for 10 min. Purified DNA was then added and the mixture was kept at 45°C for 5 min. This preincubation of the membrane fraction at 45°C was necessary for pSL103 binding to the membrane fractions isolated from both dna-1 and dna-1-1 cells (unpublished data). For the time course experiments shown in Table 3, time points were taken by diluting the sample: 1:10 in TKE buffer at 0°C. The samples were then centrifuged in sucrose gradients for 3 hr at 25,000 rpm at 4°C in an SW 27 rotor as described (6, 16, 17). The samples were divided into 25 fractions and aliquots (0.4–1 ml) were assayed for trichloroacetic acid insoluble radioactivity.

RESULTS

Binding of Exogenous pSL103 to Membrane in Vitro. We attempted to establish an in vitro DNA–membrane binding system specific for pSL103. The membrane fraction labeled with either [14C]leucine or [14C]thymine was purified from 168 trp dna-1 cells at 32°C. These cells did not harbor pSL103. Mixing the membrane fraction and the isolated [3H]pSL103 and incubating at 32°C did not lead to the formation of membrane-plasmid complex. A heat treatment was necessary. Thus, the membrane fractions were heated for 10 min at 45°C, and then purified [3H]pSL103 was added to the [14C]thymine-labeled fraction. Five minutes later, an aliquot was removed from the 45°C incubation and diluted 1:10 at 0°C. This is the 0-min incubation sample shown in Table 1. The remainder of the sample was incubated at 32°C for 30 min and then diluted as above.

To determine the number of plasmid molecules bound to the membrane fraction at 32°C, the samples were centrifuged in sucrose gradients. A typical sedimentation profile of endogenous chromosomal [14C]DNA and that of exogenously added [3H]pSL103 are shown in Fig. 1A. As shown in Fig. 1B, under the same conditions, 94% of the [14C]leucine label of the isolated membrane fraction sedimented to the previously observed membrane position, indicating that the membrane particles remain virtually intact after the reisolation procedure, which includes overnight dialysis, volume reduction with PEG 6000, and heat treatment at 45°C. However, the majority of originally membrane-bound chromosomal DNA no longer sedimented in the absence of the membrane fraction [purified pSL103 DNA sediments at the top of the sucrose gradient (17)]. Therefore, the amount of [H]labeled pSL103 found in the membrane fraction region of the gradient is proportional to the number of plasmid molecules bound to the membrane fraction at 32°C.

The number of plasmid molecules bound per cell equivalent of membrane was computed in the following manner (see also Table 1). The total number of pSL103 molecules in the gradient was calculated from the amount of [H] cpm in the gradient and the known specific activity (cpm/μg) of the plasmid. The number of cell equivalents of membrane fraction in each gradient was calculated from the original number of cells used to produce the [14C]-labeled membrane fraction and the total [14C] cpm in the gradient (Table 1). The term “cell equivalents of membrane” denotes the actual number of cells from which a specific amount of membrane fraction was prepared.

The number of plasmids bound in vitro per cell equivalent

![Figure 1](attachment:image.png)
of membrane was similar to the number found in vivo. With the assumption that the copy number per cell of pSL103 in B. subtilis is 40 (9), approximately 75% of the plasmid molecules in each cell (30 plasmids) are found in the membrane fraction of dna-i cells at 32°C.

With the assumed copy number of 40 for pSL103, the amount of plasmid added during these in vitro experiments was 2- to 3-fold in excess of the copy number. However, the degree of excess had no effect on the number of the plasmids bound, indicating that, under our binding conditions, saturation of the binding sites was reached effectively. This result suggests that the cell has a fixed capacity to accommodate pSL103. Therefore, the absolute percentage of plasmids bound to the membrane fraction is dependent upon the amount of plasmids added over saturation—i.e., under more saturating conditions the actual percentage of input plasmids bound will decrease. Because these experiments were done under saturating plasmid conditions, plasmid binding occurred rapidly. Maximal DNA binding occurred within 10 min after shifting the mixture of membrane and the plasmid to 32°C (Table 2).

Competitive Binding Studies Using ColE1 and pSL103. It was important to demonstrate that the in vitro binding of pSL103 to membrane was specific for the pSL103 molecule and not a nonspecific DNA–membrane interaction. For this purpose, the E. coli plasmid ColE1 was used as a heterologous plasmid DNA molecule. ColE1 has a molecular weight of 4.2 × 10^6 (6.4 kb) and does not replicate in B. subtilis. If the binding of pSL103 to the membrane is a nonspecific interaction, then ColE1 DNA should also bind to the membrane fraction and compete with pSL103 for membrane binding.

Membrane fraction from dna-i cells labeled with [14C]thymine was isolated and incubated at 45°C for 10 min, divided into three equal samples, and added to tubes preheated at 45°C for 10 min, containing (i) [3H]ColE1, (ii) [3H]pSL103, or (iii) [3H]pSL103 plus nonradioactive ColE1 in a molar ratio of 1:10. The samples were kept at 45°C for 5 min and then were incubated at 32°C for 30 min before dilution. The samples were centrifuged and analyzed as before.

Little or no binding of ColE1 to the membrane fraction occurred (Table 3). However, pSL103 was bound in a typical manner. When pSL103 and ColE1 were present together, the binding of pSL103 to the membrane was not affected by the presence of excess ColE1. Thus, ColE1 DNA neither bound to purified membrane fraction from B. subtilis in vitro nor competed with pSL103 for binding sites on the B. subtilis membrane. Similarly, rat liver DNA did not compete with pSL103 DNA for binding (data not shown). Some pSL103 consistently bound to the membrane even when the reaction mixture was diluted immediately after the 5-min incubation at 45°C, although at a lesser amount than that observed at 32°C (Table 3).

Under the same conditions, however, ColE1 DNA did not bind at all. Our previous result shows that some molecules of pSL103 also remain bound to the membrane in vivo at the nonpermisive temperature in dna-i cells (17). This may be due either to the binding of pSL103 to the membrane during the cooling process or to some binding of pSL103 at 45°C.

Table 1. In vitro binding of pSL103 to membrane fraction from dna-i cells

| Experiment 1 | Incubation time, min | 3H label bound, % | pSL103 bound, no./gradient | pSL103 bound, no./gradient | Cell equiv | pSL103 bound
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Membrane fraction was isolated from [14C]labeled dna-i cells grown at 32°C, concentrated to approximately 1.5 ml, and incubated at 45°C for 10 min. Purified [3H]pSL103 (approximately 10,000 cpm/μg in experiment 1 or 175,000 cpm/μg in experiment 2) was added to the membrane fraction and the mixture was allowed to incubate for 5 min at 45°C. Half of the sample was diluted 1:10 in TKE buffer and kept on ice while the other half was shifted to 32°C for 30 min and then diluted. The samples were fractionated and assayed for trichloroacetic acid-precipitable counts.

* To membrane fraction, ([3H] cpm in membrane fraction)/[3H] cpm in gradient) × 100.

1 ([3H] cpm in gradient × no. of pSL103 per μg)/[3H] cpm per μg of pSL103.

3 Number of cell equivalents of membrane fraction per gradient, = ([14C cpm in gradient × no. of cells in entire experiment)/[14C cpm in entire experiment].

4 Number of plasmids bound per cell equivalent of membrane fraction, = column 4/column 5.

Table 2. In vitro membrane binding of pSL103 at various times

| Incubation time, min | 3H label bound, % | pSL103 bound, no./gradient | pSL103 bound, no./gradient | Cell equiv | pSL103 bound
<table>
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<td>1.6 × 10^9</td>
<td>1.5 × 10^8</td>
<td>10.7</td>
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14C-Labeled membrane fraction isolated from dna-i cells was shifted to 45°C for 10 min. Purified [3H]pSL103 (48,000 cpm/μg) was added to the membrane fraction at 45°C for 5 more min, and an aliquot (time 0) was removed and diluted 1:10 in TKE buffer at 0°C. The remaining mixture was shifted to 32°C, and aliquots were removed at various time intervals and diluted.

* Column headings are as defined in Table 1.
Membrane component, inhibit or such as comparable is in cooled harboring the isolated pSL103, the brane and chromosome tion recent brane in in B. whereas bated 45°C for 10 min. An aliquot of equal volume was removed from each sample and added to a tube containing [3H]ColE1 (1,000 cpm/μg), [3H]pSL103 (5,000 cpm/μg), or [3H]pSL103 and unlabeled ColE1 in a 1:10 ratio. The tubes were incubated for 5 min more at 45°C. Half of each sample was diluted 1:10 in TE buffer and the remaining half of each sample was shifted to 32°C for 30 min and then diluted. The sample was analyzed as before.  

pUB110 competed with [3H]pSL103 for membrane binding, whereas the linear EcoRI fragment containing the trpC + gene from B. pumilus did not (Fig. 2). The lack of competition by the trpC + fragment is not a function of its conformation—in e., not a function of being circular or supercoiled (unpublished data). On the basis of these results, we conclude that the in vitro binding of pSL103 to the membrane fraction is specific for the replication vector pUB110 and not the trpC + portion of the pSL103 molecule.

DISCUSSION

The biochemistry of the initiation process of DNA replication in prokaryotic organisms is beginning to be elucidated. Although little is known about the biochemical role of the membrane in the initiation of chromosome replication in bacteria, recent evidence has shown that the DNA–membrane association is necessary in B. subtilis for the initiation of both the host chromosome and pSL103 (8). The study of these DNA–membrane interactions in vitro should prove useful in further studies on the mechanism of initiation and its regulation.

This paper reports the specific in vitro binding of a plasmid, pSL103, to the purified membrane fraction from B. subtilis. When the isolated membrane fraction from B. subtilis cells, harboring or not harboring pSL103, was heated at 45°C and cooled in the presence of exogenously added radioactive pSL103, the exogenous plasmid bound specifically to the membrane fraction. The number of plasmid molecules bound in vitro is comparable to that bound in vivo, and heterologous DNAs such as CoE1 or rat DNA do not bind to B. subtilis membrane or inhibit the binding of pSL103. These results indicate that the membrane component, presumably protein or proteins, re-

<table>
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<th>Sample</th>
<th>Incubation time, min</th>
<th>3H label bound, %</th>
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<th>Cell equiv</th>
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<td>9.4 × 10^9</td>
<td>9.2 × 10^8</td>
<td>10.3</td>
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14C-Labeled membrane fraction from dna-1 cells was incubated at 45°C for 10 min. An aliquot of equal volume was removed from each sample and added to a tube containing [3H]ColE1 (1,000 cpm/μg), [3H]pSL103 (5,000 cpm/μg), or [3H]pSL103 and unlabeled ColE1 in a 1:10 ratio. The tubes were incubated for 5 min more at 45°C. Half of each sample was diluted 1:10 in TE buffer and the remaining half of each sample was shifted to 32°C for 30 min and then diluted. The sample was analyzed as before.

* Column headings are defined as in Table 1.

**FIG. 2.** Competition among pSL103, trpC + fragment, and pUB110 for binding to membrane fraction isolated from dna-1 cells labeled with [14C]thymine. The membrane fraction was then added to tubes containing either varying amounts of covalently closed supercoiled pUB110 and supercoiled [3H]pSL103 of high specific activity (145,000 cpm/μg) or the linear EcoRI fragment coding for the trpC + gene from B. pumilus and supercoiled [3H]pSL103. The mixtures were allowed to incubate at 45°C for an additional 5 min. The samples were then shifted to 32°C for 30 min and diluted 1:10 in TE buffer at 0°C. Fraction of competitor molecules added was calculated as (number of pUB110 or of trpC + fragment)/(number of pUB110 plus number of pSL103 or number of trpC + fragment plus number of pSL103). pSL103 bound is shown per cell equivalent of membrane complex. (Inset) Map of pSL103. E, EcoRI site; ori and Nm, origin of replication and neomycin-resistance marker (locations of ori and Nm' on pUB110 as determined by Scheer-Abramowitz et al. (18); their locations relative to the B. pumilus fragment of pSL103 are not known); trpC + tryptophan C locus (exact location not known).
sponsible for the in vitro binding of pSL103 exists in the B. subtilis membrane in approximately the same amount, whether the plasmid exists in the cell or not.

The results of the competitive binding experiments in vitro demonstrate that pSL103 binds to the membrane through the pUB110 portion of this chimeric plasmid. Our results strongly suggest that DNA sequence specificity is involved in the in vitro membrane binding. It is also interesting to note that the binding is mediated by the origin-carrying fragment of pSL103 and not by the trpC' origin-carrying fragment.

Recently, we have found that there is no difference in temperature sensitivity between dna-l and dna-l' cells in the in vitro binding of the plasmid to the membrane fractions and that the in vitro complex is sensitive to salt concentrations above 0.2 M KCl (unpublished data). This is in contrast to the property of the in vivo pSL103 membrane complex we studied previously: the isolated in vivo complex from dna-l', but not the complex from dna-l', is temperature sensitive in buffer, and the complex from both strains is resistant to high salt concentration (it can be isolated in 4 M CsCl without substantial degradation) (17). These results indicate that the specific in vitro complex we describe here is only a part of the complete picture of the origin-membrane complex in vivo. Because of the specificity of the binding, it is unlikely that the complex formation in vitro is irrelevant to the in vivo situation. The critical question is whether the type of complex described in this paper exists in the cell in addition to the high-salt-resistant complex or whether the in vitro high-salt-sensitive complex corresponds to a pre-mature complex which becomes the high-salt-resistant one by further processes in vivo.

The reason for the necessity of heat treatment at 45°C of the mixture of membrane and plasmids for the in vitro binding is not clear. Some conformational change of a membrane component or the plasmid may be necessary for the binding to occur. In vivo, some factor probably substitutes for the high temperature.

An in vitro site-specific binding of DNA to an isolated membrane protein (protein B') has been reported in E. coli; the protein binds to two sites flanking the chromosome origin (19). The binding is different from the in vitro pSL103 membrane binding in B. subtilis reported here in that the E. coli origin DNA binds to the protein B' in the single-stranded form only, whereas pSL103 or pUB110 binds to the B. subtilis membrane in the double-stranded form.

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