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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-1*, 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*$^+$-carrying portion neither binds to the membrane fraction nor competes with pSL103 for binding to the membrane fraction *in vitro*. ColE1 plasmid, which does not replicate in *B. subtilis*, neither binds to the *B. subtilis* membrane fraction nor competes with pSL103 for binding.**

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-1* 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-1* 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-1* 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-1* and *dnaB19* carry defects in distinct but closely linked loci, *dnaBI* and *dnaBII*, respectively (13). When purified DNA–membrane complex isolated from *dna-1* (*dnaBI*) 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-1* mutation was grown at 32°C in medium supplemented with thymine at 5 μg/ml. The cells were grown to 7×10^7 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 [^{3}H]-thymidine (50 Ci/mmol; 1 Ci = 3.7×10^{10} Bq; Amersham) at 4 μCi/ml, [^{14}C]thymine (50 mCi/mmol; New England Nuclear) at 1 μCi/ml, or [^{14}C]leucine (50 mCi/mmol; New England Nuclear) at 1 μCi/ml. ^{3}H-Labeled plasmid prepared by growing 168 *trp thy* cells harboring the plasmid with [^{3}H]-thymidine as above had a maximal specific activity of 3.4×10^4 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 7×10^7 cells per ml (40 Klett units), [^{3}H]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 [^{3}H]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 3×10^5 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.

Plasmid 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

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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 37°C, 2.5 ml of 0.25 M EDTA (pH 8.0) was added and the cells were 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 30 fractions, and 0.015 ml was removed from each fraction for assay of radioactivity. The pooled DNA fractions containing plasmid were treated as 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 mM 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*$^+$ 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 [^{14}C]leucine or [^{14}C]thymine was purified from 168 *trp thy dna-1* cells at 32°C. These cells did not harbor pSL103. Mixing the membrane fraction and the isolated [^{3}H]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 [^{3}H]pSL103 was added to the [^{14}C]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 [^{14}C]DNA and that of exogenously added [^{3}H]pSL103 are shown in Fig. 1*A*. As shown in Fig. 1*B*, under the same conditions, 94% of the [^{14}C]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 ^{3}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 *in vitro*.

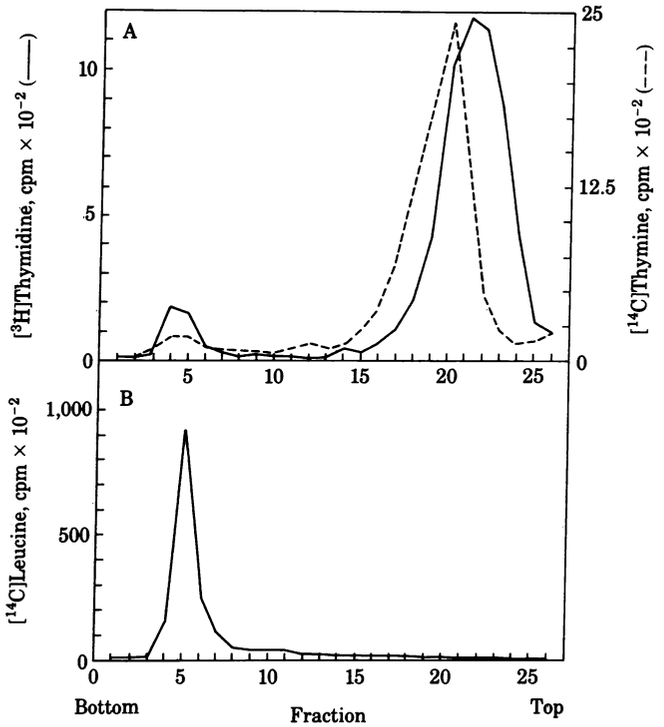

FIG. 1. (*A*) *In vitro* binding of exogenous [^{3}H]pSL103 to the isolated membrane fraction. Membrane fraction was isolated from a 25-ml culture of 168 *thy trp dna-1* cells labeled at 32°C with [^{14}C]thymine (1 μCi/ml). The fraction was dialyzed overnight against H_2O at 4°C and concentrated to approximately 1.5 ml by dialysis against polyethylene glycol 6000 (PEG 6000). The membrane fraction was heated at 45°C for 10 min. Purified [^{3}H]pSL103 (10,000 cpm/μg) was added to the membrane fraction in saturating amounts (approximately 2,500 molecules per cell equivalent of membrane fraction) at 45°C for 5 min longer. The sample was shifted to the permissive temperature (32°C) for 30 min and diluted 1:10 in TKE buffer at 0°C. (The temperature of the diluent had no effect on the results—i.e., dilution at 32°C gave identical results.) The samples were layered on a 5–20% linear sucrose gradient with a 62% sucrose shelf and centrifuged at 20,000 rpm at 4°C for 3 hr in a Beckman SW 27 rotor. The gradients were fractionated into 25 tubes (approximately 1.2 ml per tube) from the bottom of the gradient with a peristaltic pump. The entire sample was assayed for trichloroacetic acid-precipitable counts. The solid line is the [^{3}H]plasmid profile. The dashed line represents the [^{14}C]thymine profile for the host DNA. (*B*) Sedimentation profile of membrane fraction isolated from *dna-1* cells grown in the presence of [^{14}C]leucine. [^{14}C]Leucine-labeled membrane fraction was isolated by sucrose gradient centrifugation, incubated at 45°C, and centrifuged again in exactly the same way as the thymine-labeled sample in *A*.

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 ^{3}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 ^{14}C-labeled membrane fraction and the total ^{14}C 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

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

Incubation time, min	^{3}H label bound,* %	pSL103 added, no./gradient†	pSL103 bound, no./gradient‡	Cell equiv§	pSL103 bound¶
		Experiment 1			
0	1.2	1.2×10^{11}	1.4×10^{9}	8.6×10^{7}	16.3
30	2.6	1.3×10^{11}	3.4×10^{9}	9.8×10^{7}	34.7
		Experiment 2			
0	10.6	5.2×10^{10}	5.5×10^{9}	3.7×10^{8}	14.9
30	15.8	7.9×10^{10}	1.2×10^{10}	4.9×10^{8}	24.5

Membrane fraction was isolated from ^{14}C-labeled *dna-1* cells grown at 32°C, concentrated to approximately 1.5 ml, and incubated at 45°C for 10 min. Purified [^{3}H]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, (^{3}H cpm in membrane fraction/^{3}H cpm in gradient) × 100.

† (^{3}H cpm in gradient × no. of pSL103 per μg)/^{3}H cpm per μg of pSL103.

‡ (Column 2 × column 3)/100.

§ Number of cell equivalents of membrane fraction per gradient, = (^{14}C cpm in gradient × no. of cells in entire experiment)/^{14}C cpm in entire experiment.

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

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-1* 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-1* cells labeled with [^{14}C]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*) [^{3}H]ColE1, (*ii*) [^{3}H]pSL103, or (*iii*) [^{3}H]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 nonpermissive temperature in *dna-1* 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.

Binding of pSL103 Is Effected Through pUB110 DNA Fragment. The pSL103 plasmid is a chimera of the *S. aureus* plasmid pUB110 and an *Eco*RI fragment from *B. pumilus* containing the *trpC*$^+$ gene (9). Because pUB110 provides the rep-

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

Incubation time, min	^{3}H label bound, %	pSL103 added, no./gradient	pSL103 bound, no./gradient	Cell equiv	pSL103 bound
0	3.6	1.6×10^{10}	6.1×10^{8}	1.4×10^{8}	4.4
10	13.0	1.2×10^{10}	1.6×10^{9}	1.4×10^{8}	11.4
20	13.0	1.2×10^{10}	1.6×10^{9}	1.6×10^{8}	10.0
30	13.5	1.2×10^{10}	1.6×10^{9}	1.5×10^{8}	10.7

^{14}C-Labeled membrane fraction isolated from *dna-1* cells was shifted to 45°C for 10 min. Purified [^{3}H]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.

Table 3. Competition of ColE1 with pSL103*

Sample	Incubation time, min	^{3}H label bound, %	pSL103 bound, no./gradient	Cell equiv	pSL103 bound
		Control			
[^{3}H]ColE1	0	0:16	5.5×10^8	6.2×10^9	0.08
	30	0.31	5.6×10^8	6.2×10^9	0.09
		Experiment 1			
[^{3}H]pSL103	0	13.4	8.8×10^{10}	4.8×10^9	18.0
	30	19.4	1.4×10^{11}	5.7×10^9	24.5
[^{3}H]pSL103 + ColE1	0	16.9	9.9×10^{10}	6.2×10^9	15.0
	30	23.0	1.6×10^{11}	5.9×10^9	29.3
		Experiment 2			
[^{3}H]pSL103	0	18.0	4.6×10^9	8.4×10^8	5.4
	30	27.0	3.0×10^{10}	1.4×10^9	21.3
[^{3}H]pSL103 + ColE1	0	19.0	4.1×10^9	1.4×10^9	2.9
	30	26.0	9.4×10^9	9.2×10^8	10.3

^{14}C-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 [^{3}H]ColE1 (1,000 cpm/μg), [^{3}H]pSL103 (3,000 cpm/μg), or [^{3}H]pSL103 and nonlabeled 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 TKE 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 as defined in Table 1.

lication vector for pSL103, it may compete for membrane binding with pSL103, whereas the *trpC*$^+$ fragment may not. To test this possibility, isolated membrane fraction was incubated at 45°C for 10 min and divided equally into tubes containing either various amounts of covalently closed circular pUB110 and [^{3}H]pSL103 molecules or the EcoRI *trpC*$^+$ fragment and covalently closed circular [^{3}H]pSL103. The samples were incubated for 5 min more at 45°C. Each tube was then shifted to 32°C for 30 min and diluted. The samples were analyzed as before.

pUB110 competed with [^{3}H]pSL103 for membrane binding, whereas the linear *Eco*RI 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—i.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 (6). 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 ColE1 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-

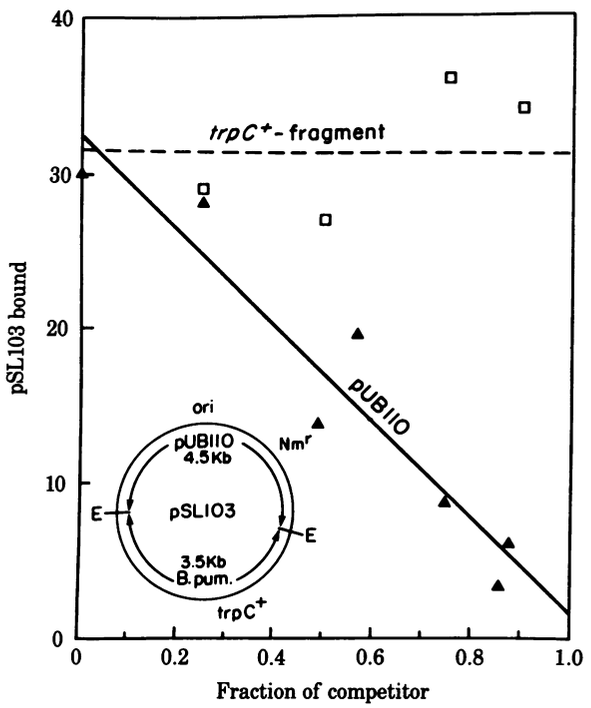

FIG. 2. Competition among pSL103, *trpC*$^+$ fragment, and pUB110 for binding to membrane fraction isolated from *dna-1* cells labeled with [^{14}C]thymine. The membrane fraction was then added to tubes containing either varying amounts of covalently closed supercoiled pUB110 and supercoiled [^{3}H]pSL103 of high specific activity (145,000 cpm/μg) or the linear *Eco*RI fragment coding for the *trpC*$^+$ gene from *B. pumilus* and supercoiled [^{3}H]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 TKE 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, *Eco*RI site; ori and Nm^r, origin of replication and neomycin-resistance marker [locations of ori and Nm^r 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*$^+$-carrying fragment.

Recently, we have found that there is no difference in temperature sensitivity between *dna-1* and *dna-1*$^+$ 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-1*, but not the complex from *dna-1*$^+$, 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 premature 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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