JOURNAL OF BACTERIOLOGY, Dec. 1991, p. 7732–7735
0021-9193/91/237732-04$02.00/0

Vol. 173, No. 23

In Vitro Type II Binding of Chromosomal DNA to Membrane in *Bacillus subtilis*

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Received 17 June 1991/Accepted 26 September 1991

DNA-membrane association critical for initiation of DNA replication in *Bacillus subtilis* can be classified into two types. Type I is salt resistant and dependent on the initiation gene, *dnaB*, and type II is salt sensitive and independent of the *dnaB* gene. We found and sequenced two adjacent areas of type II binding within 1% of *oriC* on the *B. subtilis* chromosome.

A number of studies have demonstrated an association between the origin and terminus areas of the bacterial chromosome and the plasma membrane (for reviews, see references 1 and 11). The origin attachment of the bacterial chromosome may play two roles; one is a regulatory role of the initiation of chromosome replication at *oriC*, and the other is a physical role in the partition of two daughter chromosomes (10, 11).

We have reported two types of membrane binding in pUB110, type I and type II. Type I binding is salt resistant and was first discovered in the *oriC* area of *Bacillus subtilis* (8). Type I binding was subsequently shown to be dependent on the function of the *dnaB* gene, which had previously been known as an initiation gene essential for both the *B. subtilis* chromosome and the plasmid pUB110 (10). Type II binding was found by an in vitro membrane-binding assay (3) using the chimeric plasmid pSL103 (2). Type II binding was found only in the pUB110 part of pSL103 and was localized to four distinct areas of the pUB110 molecule. The binding was salt sensitive and independent of the function of the *dnaB* gene (9). The results of previous experiments (3, 9) have raised the possibility that there may be chromosomal type II membrane-binding sites as well. To investigate this possibility, we have examined DNA-membrane interactions using the plasmid pAZ108, which contains *B. subtilis* DNA from near the *purA16* region. Previous studies have indicated that the *purA16* region is associated with proteins in a soluble or S complex (12). Sargent and Bennett (5, 7) have reported that the same region described by Yamaguchi and Yoshikawa (12) as part of the S complex is membrane bound when nucleoids are digested by restriction enzymes and then fractionated. Furthermore, they have indicated that this association is through a sequence of DNA contained within a 3.6-kb *Eco*RI fragment adjacent to the *purA16* locus (6). We have investigated the association between membrane complexes isolated from cell lysates and labeled DNA fragments by using in vitro DNA-membrane binding conditions that are specific for the type II membrane binding (3, 9).

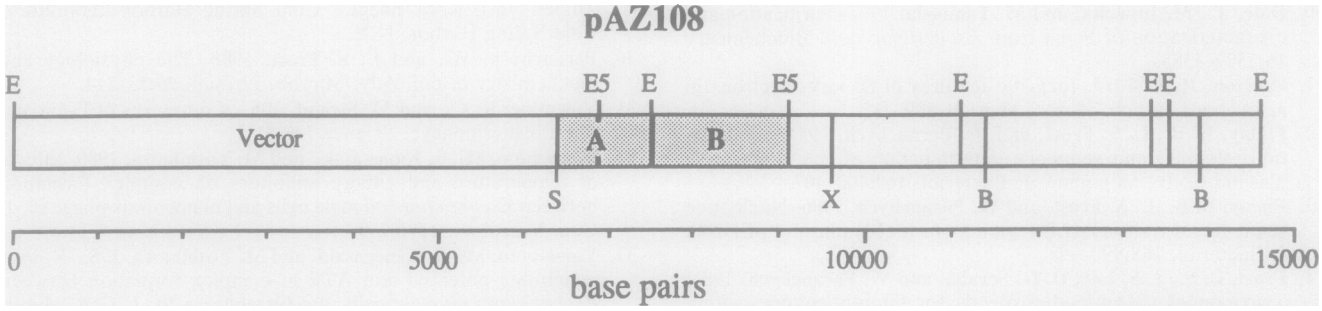

FIG. 1. Restriction map of the plasmid pAZ108, which contains a DNA fragment of the *B. subtilis* chromosome carrying the type II membrane areas A and B. pAZ106 is the original vector used for subcloning this region. The shaded areas (A and B) represent the fragments that show binding by the type II binding assay defined by Tanaka and Sueoka (9). B, *Bgl*II; E, *Eco*RI; S, *Sst*I; X, *Xba*I; E5, *Eco*RV.

Construction of the plasmid pAZ108. *Eco*RI-digested *B. subtilis* chromosomal DNA fragments were subcloned into pAZ106 (a 8.35-kb derivative of pBR322; Amp^r Tet^s, with the *lacZ-erm* gene construct from the Tn*917-lac* transposon [13]). One of the recombinant plasmids (pAZ104) carried a 1.1-kb *Eco*RI *B. subtilis* DNA fragment that was used to do chromosome walking, picking up in the process an additional 6.7 kb of *B. subtilis* DNA. This new plasmid, containing 7.8 kb of *B. subtilis* DNA, was designated pAZ108. Chromosomal DNA or DNA that was included within the plasmid pAZ108 was digested with various restriction enzymes. Southern hybridizations were performed with various probes to generate the restriction map shown in Fig. 1.

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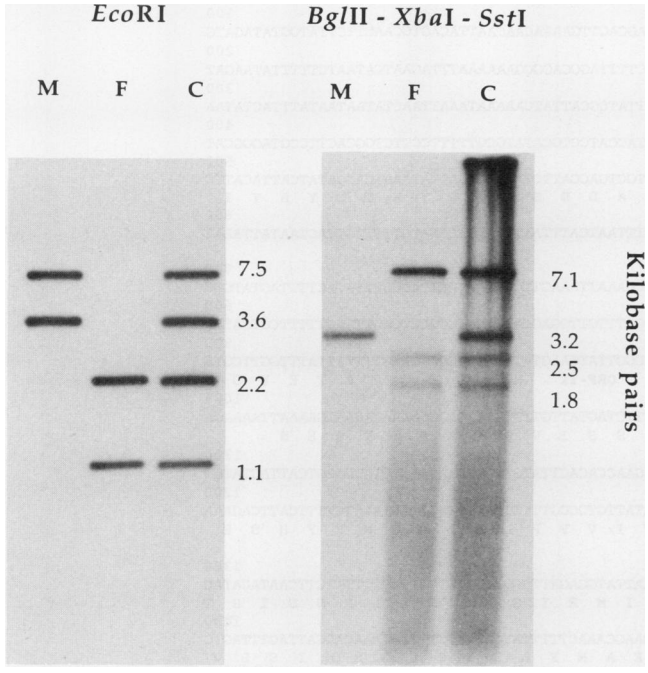

FIG. 2. The results of type II binding assays run on 1.2% agarose gels. The left three lanes are *Eco*RI-digested and end-labeled pAZ108 DNA. The right three lanes contain the same preparation digested with *Bgl*II-*Xba*I-*Sst*I. Lane C has labeled DNA only, lane F is DNA recovered from the sucrose gradient-free fraction, and lane M has DNA from the membrane fraction. The smallest *Eco*RI fragment, of 0.2 kb (Fig. 1), has run off of the gel in this figure. In separate experiments, the 0.2-kb fragment did not bind to the membrane fraction (result not shown). The small size of the 0.2-kb fragment cannot be the reason for the lack of membrane binding, because we have previously shown that a 0.23-kb fragment of pUB110 showed a strong type II binding (9). Also, a larger fragment which included the 0.2-kb fragment did not bind. The exclusive binding of fragments A and B (Fig. 1) has been obtained with up to ~200 copies per cell equivalent. With more than 200 copies per cell equivalent, unbound A and B fragments begin to be found in the free fraction (data not shown).

Binding assay. Cell lysates of *B. subtilis* 168TT or 168TT *dna-1* were prepared as described by Tanaka and Sueoka (9). The binding was done as described previously (9). Briefly, the frozen cell pellets were resuspended in 0.6 ml of buffer (20 mM Tris-HCl, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mg of lysozyme per ml, and 1 mM 2-mercaptoethanol). After 20 min at 32°C, 0.1 ml of 5% Brij-58 was added to the mixture. After 2 min at room temperature, the protoplasts were sheared gently through an 18-gauge needle five times. Samples (200 μl) were incubated at 45°C for 10 min (3). Twenty microliters of ^{32}P end-labeled DNA fragments was added to the lysates, which remained at 45°C for an additional 5 min. The reaction mixtures were transferred to 32°C for 20 min and then placed on ice. Reaction mixtures were separated on a 20 to 5% linear sucrose gradient as described previously (9). Membrane and free fractions were separately pooled and phenol extracted; they were then ethanol precipitated, and isolated DNA fragments from each fraction were resuspended and assayed for radioactivity. Appropriate amounts of each sample for equal radioactive counts were electrophoresed on 1.2% agarose gels that were vacuum dried and then autoradiographed.

By digesting pAZ108 DNA with various restriction endonucleases before they were end labeled and added to the binding assays, we have observed that the binding areas of the plasmid are limited to the area from the *Sst*I site to the *Xba*I site. Further digestion of the plasmid with *Eco*RI prior to use in the binding assays shows that two fragments, 7.5 kb and 3.6 kb, bind to the membrane (Fig. 2). The results indicate that the two areas are independently capable of binding to membrane. The plasmid pAZ108 has two *Eco*RV sites, one in each of the binding fragments. If the plasmid is digested with both *Eco*RI and *Eco*RV, only the B fragment (1,577 bp) still binds to membrane. The minimal areas required for binding to membrane in our type II binding assay are from *Sst*I to *Eco*RI for fragment A and from *Eco*RI to *Eco*RV for fragment B.

The results presented in this work clearly demonstrate that a complex in addition to the previously known the DNA-membrane complex (type I) is formed between specific fragments of origin area DNA and membrane. The complex isolated by our in vitro experiments shows two adjacent DNA fragments with characteristics similar to those of type II membrane binding, originally discovered in pUB110 by Korn et al. (3). One of the two type II-binding areas observed in this study coincides with the in vivo membrane-bound area isolated by Sargent, Bennett, and Burdett (5, 7) in which DNA-membrane complex was isolated after digestion of nucleoids with *Eco*RI. In combination, these results from the two laboratories lead to two conclusions. (i) The membrane-binding area of the *B. subtilis* chromosome reported by Sargent and Bennett is type II binding and (ii) the type II binding originally demonstrated by in vitro reconstruction experiments in pUB110 does exist in vivo in the *B. subtilis* chromosome as well. In addition to the specific fragment reported by Sargent and Bennett (6), we have shown that there is another independent type II-binding area adjacent to the one reported (fragment B in Fig. 1). The exact position of this type II-binding area has been mapped to the physical chromosome by M. Itaya (personal communication). It is located 37 kb counterclockwise from the *oriC* assigned by Ogasawara et al. (4).

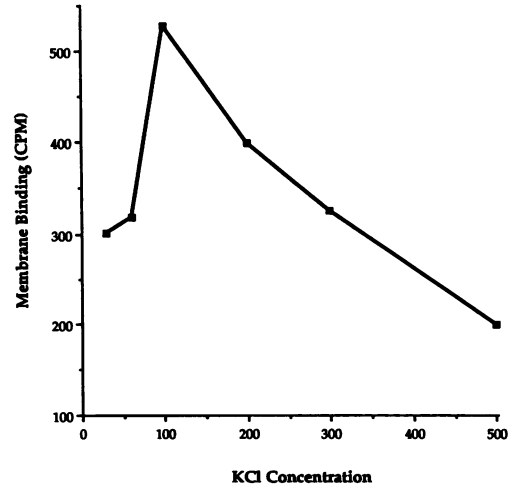

FIG. 3. A salt sensitivity test of type II binding of *Eco*RI-cut pAZ108. Cell lysates were prepared as described previously. Aliquots of 100 μl were diluted with 100 μl of the appropriate KCl solution to yield final concentrations ranging from 30 to 500 mM KCl. Radioactive DNA samples were added, and the assay was completed as described above. Total counts per minute (cpm) in each membrane fraction were calculated and graphed.

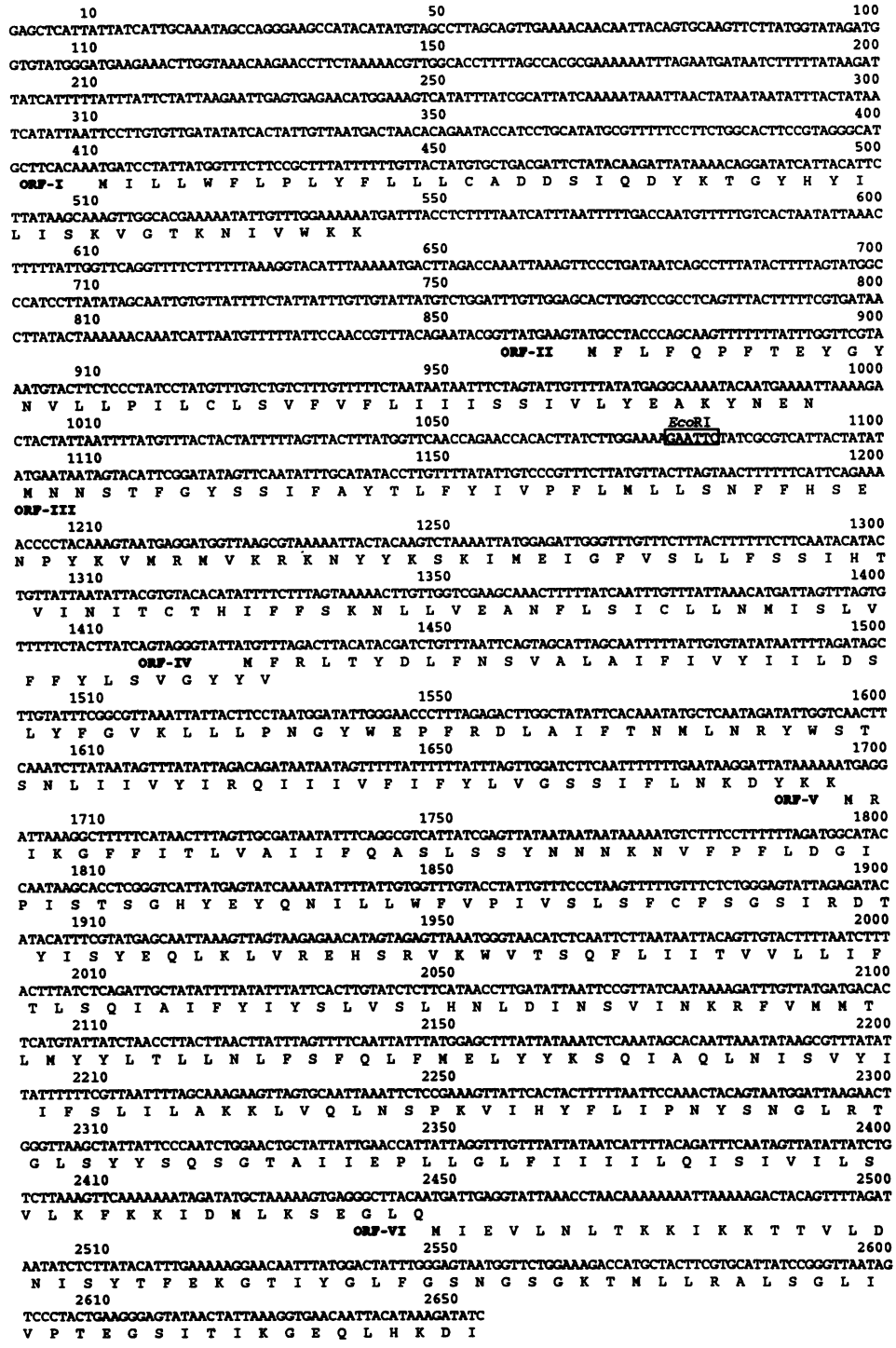

FIG. 4. The DNA sequence of pAZ108 from the *Sst*I site to the second *Eco*RV site (Fig. 1). All open reading frames larger than 40 amino acids are in the same orientation. The six possible open reading frames are shown (labeled ORF-I through ORF-VI). The boxed region shows the *Eco*RI site that divides the two binding regions. Binding area A corresponds to nucleotides 1 to 1078 (the *Eco*RI box). Binding area B corresponds to the area from the *Eco*RI box to the end of the sequence.

Salt sensitivity of membrane binding. To determine the effect of various salt concentrations on the association between DNA and membrane, the standard cell lysate was prepared as described earlier. Aliquots were diluted with an appropriate KCl solution to yield final concentrations ranging from 30 to 500 mM KCl. Radioactive DNA samples were added, and the assay was completed as described above. Following fractionation of lysates on sucrose gradients, total counts per minute in each membrane fraction with different concentrations of KCl were calculated and graphed. Figure 3

shows that the optimal association between membrane and DNA fragments is found with 100 mM of KCl. In contrast to type I binding, which resists 5 M CsCl (8, 10), this salt sensitivity is comparable to the optimal value that has previously been observed for the type II binding of pUB110 (60 mM) (9).

Sequence data. DNA from the plasmid pAZ108 was cut with several restriction enzymes. Subclones of the insert DNA within the plasmid (Fig. 1) were constructed by using either M13 or pUC vectors, and the entire area was sequenced in both directions. The sequence of the 2.7-kb region from the *Eco*RV site to the *Sst*I site is shown in Fig. 4. The sequence from this *Eco*RV site to the *Ava*I site at position 1,811 of our sequence is most likely the one published previously by Sargent and Bennett (6). The sequences determined by the two laboratories mostly agree. The reason for the minor discrepancies is currently not clear. Nevertheless, because of the agreement of 2,644 nucleotides out of 2,653, we conclude that we are dealing with the same area of the chromosome.

Both of the fragments from this region that bind to membrane in our type II assay have high AT contents. When the nucleotide sequences of these two fragments are compared, several areas of high homology stand out. These areas are almost entirely composed of A and T. The type II-binding fragments of pUB110 also have regions of high AT content. Exactly what significance these AT-rich regions have is unknown at this time.

The minimal region necessary for recovery in the particulate fraction as described by Sargent and Bennett (6) was from the *Msp*I site (nucleotide 2589 of our sequence, Fig. 4) to the *Alu*I site (nucleotide 2304, Fig. 4). The region we describe for binding in our type II experiment also includes this area. The fragment described by Sargent and Bennett (6) and the fragment we describe here must therefore be the same. Further work remains to determine how similar the two complexes are and to pinpoint the protein(s) responsible for the sequence-specific binding.

Nucleotide sequence accession number. The DNA sequence reported here has been submitted to GenBank and has been assigned the accession number M 77837.

We are grateful to M. Itaya for mapping the position of the binding sites.

This work has been supported by NIH grant GM 28133 and NSF grant DMB 8905762.

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