JOURNAL OF BACTERIOLOGY, Apr. 1980, p. 339-343
0021-9193/80/04-0339/05$02.00/0

Vol. 142, No. 1

Membrane Association of a *Staphylococcus aureus* Plasmid in *Bacillus subtilis*

SCOTT WINSTON AND NOBORU SUEOKA*

Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309

The *Staphylococcus aureus* plasmid pUB110 was found to be enriched in deoxyribonucleic acid-membrane complexes isolated from *Bacillus subtilis* containing pUB110.

In *Bacillus subtilis*, the replication origin (2, 11, 16, 18, 19, 22), the replication terminus (16, 18, 19, 24), and the replication fork (8, 11, 23) have been shown to be preferentially associated with the cell membrane. A physiological role for these specific DNA-membrane complexes has not yet been demonstrated, and the elucidation of their role has been hindered by the large size of the bacterial chromosome and nonspecific DNA-membrane associations.

To understand the functional role of DNA-membrane complexes, we have examined the DNA-membrane association of the *Staphylococcus aureus* plasmid pUB110 with the hope that a small replicon might serve as a model system. The plasmid pUB110 replicates in *B. subtilis* at a copy number of 30 to 50 (7), expresses resistance to neomycin (Nm^r) and kanamycin (7), and requires some of the *B. subtilis dna*$^+$ gene products for replication (15). For most of the studies reported here, a recombinant plasmid, pSL103, was used (12). The plasmid pSL103 consists of pUB110, which is necessary for replication, and a single *Eco*RI fragment from *Bacillus pumilus*, which can complement but not transform *trpC* mutations of *B. subtilis* to *trpC*$^+$ (12). As described below, the presence of the *trp*$^+$ fragment did not affect the membrane association of pUB110.

Two separate methods of preparing DNA-membrane complexes were used to study the DNA-membrane association of pSL103. The first method separated membrane-bound and membrane-free DNA by centrifugation in linear CsCl-sucrose double gradients (19), and the second method separated the two DNA fractions in sucrose gradients (25). The CsCl-sucrose gradients are preformed linear gradients of 5 to 0.5 M CsCl and 20 to 5% sucrose. The membrane-associated DNA and free DNA sediment to equilibrium within 30 min. This method separated the DNA-membrane fraction into two distinct complexes, M_1 and M_2. The two complexes are nearly identical in their enrichment for genetic markers near the origin and terminus, but are different in their protein composition and DNA-to-protein ratio (10, 19, 22).

DNA-membrane complexes were isolated from *B. subtilis* BR151 *lys*(pSL103) cells (12) uniformly labeled with [^{3}H]thymidine by centrifugation in CsCl-sucrose gradients (19). Plasmid was detected by its ability to transform competent *B. subtilis* cells to Nm^r. Membrane enrichment indices (MEI) were then calculated for pSL103; a chromosomal origin marker, *purA16*; and a terminus marker, *metB5* (18; footnote to Table 1). The marker *leuA8*, which is not enriched in the DNA-membrane fraction, was used as the standard marker for normalization of the enrichments of the other markers (18). An MEI greater than 1 indicates enrichment of that marker in the DNA-membrane fraction.

As seen in Table 1, pSL103 is more enriched in both M_1 and M_2 than the chromosomal origin and terminus. Nearly identical results were obtained from BR151 *lys* cells containing pUB110 (Table 1, in parentheses), suggesting that the pUB110 portion of pSL103, and not the *trpC*$^+$ fragment, is responsible for plasmid-membrane association. The plasmid pSL103 was also enriched in the DNA initiation mutant of *B. subtilis dna-1 trp thy* (20) at the permissive temperature of 32°C (Table 1). Since these and previous results (19, 22) have shown that M_1 and M_2 are similarly enriched for the plasmid and the chromosomal origin and terminus, we have combined M_1 and M_2 and considered them as the DNA-membrane fraction (M fraction) from which they were derived. MEIs calculated by combining M_1 and M_2 are shown in Table 1. These results demonstrate the enrichment of pSL103 in DNA-membrane fractions.

Transformation by plasmid DNA in *B. subtilis* has been shown to be dependent on plasmid DNA conformation (5). Oligomeric plasmid species transformed 100 times more efficiently than monomeric plasmid, but only 25% of the total

TABLE 1. *MEIs[a] calculated from membrane complexes isolated from CsCl-sucrose gradients*

B. subtilis strain	MEI			% pSL103
	Nm^r	*purA16*	*metB5*	
BR151 *lys*				
M_2	7.4 (7.2)[b]	3.5 (2.4)	1.7 (1.4)	
M_1	6.2 (6.6)	2.0 (2.1)	1.5 (1.4)	
168 *dna-1 trp thy*[c]				
M_2	8.4	1.8	1.4	41
M_1	6.8	2.0	3.0	36
168 *dna-1 trp thy*[d]				
$M_1 + M_2$	7.2	2.3	2.2	62
Recentrifugation of *d*				36

[a] *B. subtilis* BR151 *lys* containing pSL103 (a spontaneous revertant of BR151 *lys met* [12]) was grown in 5 ml of SPC$^+$ at 37°C (1) supplemented with 50 μg of L-lysine per ml and containing [^{3}H]thymidine (5 μC/ml specific activity, 40 to 60 Ci/mmol) and 5 μg of neomycin per ml. At Klett 40 (7×10^7 cells per ml), the cells were harvested and lysed; and layered on 4.4-ml linear CsCl-sucrose gradients as described previously (19). After centrifugation in a SW50.1 rotor for 30 min at 35,000 rpm at 4°C, the gradients were fractionated and assayed for radioactivity (20). M and F DNA fractions were pooled, and the DNA was purified (2). For the recentrifugation experiments, pooled M fractions were first dialyzed against SSC (0.1 M NaCl–0.015 M sodium, pH 7.0) and then centrifuged under identical conditions to the first centrifugation. The DNA was then used to transform competent *leuA8 metB5 purA16* by the method of Bott and Wilson (4). Nm^r transformants were selected after a 90-min expression by plating on tryptose blood agar plates containing 5 μg of neomycin per ml. Transformants for the auxotrophic markers were selected as before (21). MEIs were calculated by the expression MEI = $(X_M/X_F)/(S_M/S_F)$, where X = the number of Nm^r, *purA16*, or *metB5* transformants in the M or F fraction, and S = the number of *leuA8* transformants (16). The percent pSL103 was calculated from the total percentage of Nm^r transformants in the appropriate fraction.

[b] Numbers in parentheses identical to *a*, except BR151 contained pUB110.

[c] Identical to *a* except *dna-1 trp thy* was grown at 32°C, and the medium was supplemented with 5 μg of thymine per ml.

[d] Identical to *c* except the M_1 and M_2 fractions were pooled into one M fraction.

plasmid DNA in *B. subtilis* was found to be in oligomeric forms (5). An MEI for pSL103 calculated by transformation may then have actually demonstrated only an enrichment of oligomeric plasmids, and not total plasmid DNA, in the M fraction. To determine whether the M fraction was enriched in total plasmid DNA, the relative amount of plasmid in both the M and free (F) fractions was determined either by fluorography or by hybridization. By determining with ethidium bromide fluorography or hybridization the relative amounts of plasmid DNA in the M and F fractions to the amounts of total DNA in the M and F fractions, we could determine whether the plasmid was enriched in one fraction or the other.

DNA was purified by phenol extraction from M and F fractions of *B. subtilis dna-1 trp thy* cells containing pSL103, treated with *Eco*RI, and electrophoresed through a 0.7% agarose gel (9). The plasmid pSL103 has two *Eco*RI sites, and cleavage by *Eco*RI results in the production of two fragments, pUB110 and a trpC$^+$ fragment (Fig. 1). The DNA was transferred to a nitrocellulose filter by the Southern method (17), and ^{32}P-labeled pSL103 prepared by nick translation (14) was hybridized to the filter (6) (Fig. 1). The relative amount of pSL103 DNA in the M and F fractions was determined by densitometer tracings either of the negative of the fluorograph of the gel (Fig. 1a and b) or of X-ray film exposed to the Southern filter of the gel (Fig. 1c and d). The time of exposure was kept within the linear response range of the X-ray film. MEIs were then calculated by normalizing the relative amount of plasmid DNA in each lane to the total amount of DNA electrophoresed in each lane. The relative intensity of the fluorescent bands is directly proportional to the amount of DNA. Also, it has previously been demonstrated that the Southern hybridization technique can be quantitative, and the autoradiographic response is a direct measurement of the amount of DNA present (3, 13).

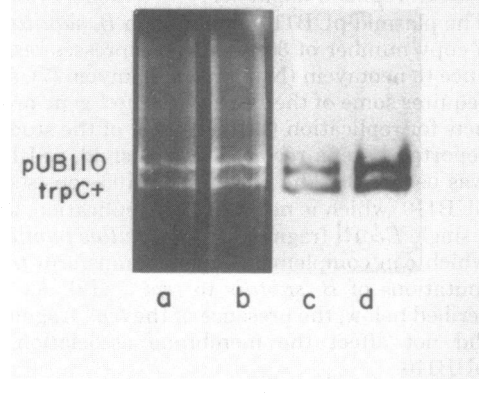

FIG. 1. *Determination of membrane enrichment by hybridization and fluorography. DNA from M and F fractions from CsCl-sucrose gradients was treated with EcoRI (9), electrophoresed through an agarose gel (9), and transferred to a nitrocellulose filter (17), as described in the text. ^{32}P-labeled pSL103 was hybridized to the filter, and the filter was exposed to Kodak XR-5 film for 1 day. The relative intensity of each band was determined by densitometer tracing with a Gelman densitometer. (a) DNA from M fraction visualized by ethidium bromide fluorescence; (b) DNA from F fraction visualized by ethidium bromide fluorescence; (c) hybridization of ^{32}P-labeled pSL103 to a; (d) hybridization of ^{32}P-labeled pSL103 to b. Total DNA includes both chromosomal and plasmid DNA. The chromosomal DNA appears as the faint fluorescent background in a and b.*

As seen in Table 2, the pSL103 MEI determined by transformation was similar to the MEI calculated by either fluorography or hybridization of pSL103. The fact that the results obtained by hybridization were similar to those obtained by fluorography indicates the Southern hybridization was quantitative under our conditions. Similar results were obtained with other samples (data not shown), demonstrating that the calculation of an MEI for pSL103 by transformation was an accurate measurement of the total plasmid enrichment in the M fraction.

Sueoka and Hammers (19) previously demonstrated that determining membrane enrichments by normalizing the number of transformants of the marker in question to the amount of transforming DNA resulted in similar results to those obtained relative to the number of *leuA8* transformants. The MEIs for *purA16* and *metB5* calculated relative to the total amount of ^{3}H-labeled transforming DNA were also similar in our experiment to the MEIs calculated relative to *leuA8* transformants (Table 2). In summary, MEIs calculated by relating either hybridization or transformation values to the amount of labeled DNA gave nearly identical results to those MEIs determined by relating the number of transformants for one marker to the number of transformants for *leuA8*.

To test for any artifactual aggregation of pSL103 with the membrane during cell lysate preparation, 0.5 μg of exogenous ^{3}H-labeled pSL103 was added during lysis to cells uniformly labeled with [^{14}C]thymine. After centrifugation in CsCl-sucrose gradients, 28% of the ^{14}C-label was found in both M_1 and M_2, whereas only 2.2% of the ^{3}H-labeled plasmid was found in those same fractions. Thus, the association of the plasmid observed here with the membrane was not found to be artifactual.

TABLE 2. *Comparison of MEIs calculated by transformation, hybridization, and fluorography*

Membrane enrichment	MEI		
	Nm^r	*purA16*	*metB5*
Relative to *leuA8*	6.1	4.1	1.9
Relative to [^{3}H]DNA	5.8[a]	4.7[b]	2.2[b]
Relative to [^{3}H]DNA	6.8[c]		

[a] MEI = $(H_M/H_F)/([^3H]DNA_M/[^3H]DNA_F)$, where H was the relative percent hybridization determined by densitometer tracings of Fig. 1c and d, and [^{3}H]DNA was the total amount of DNA loaded on the gel in each well. The DNA was uniformly labeled with [^{3}H]thymine during growth, as described in the legend to Fig. 2. A total of 463 (M) and 3,845 (F) cpm of [^{3}H]DNA was electrophoresed.

[b] MEI = $(X_M/X_F)/([^3H]DNA_M/[^3H]DNA_F)$, where *X* was the number of *purA16* or *metB5* transformants and [^{3}H]DNA was the total amount of donor DNA added to the competent cells.

[c] Same as *a* except the relative amount of plasmid DNA (*H*) was determined by densitometer tracing of the fluorograph (Fig. 1a and b).

To study further the DNA-membrane association of pSL103, we employed a second method of DNA-membrane isolation. Yamaguchi and Yoshikawa (25) reported the isolation of a membrane (M) and a soluble (S) DNA complex with sucrose density gradients. The M_1 and M_2 complexes found in CsCl-sucrose gradients are derived from M complexes isolated by sucrose gradient centrifugation (18). The S complex was shown to be a specific DNA-protein complex containing only DNA near the *purA16* loci and several unique proteins, lipid, and RNA (26).

M and S complexes were isolated from BR151 *lys*(pSL103) and *dna-1 trp thy*(pSL103) cells uniformly labeled with [^{3}H]thymidine, as described by Yamaguchi and Yoshikawa (25). A modification was made in the centrifugation procedure so that M and S complexes were isolated in one step (Fig. 2). Cell lysates were layered on sucrose gradients and centrifuged for 3 h at 20,000 rpm. This allowed the S complex to sediment away from the free DNA, but the force was not sufficient to sediment the S complex into the more dense M complex, which sedimented to the top of the 64% sucrose shelf.

The MEI for the plasmid and chromosomal markers in the M and S complexes are shown in Table 3. The plasmid pSL103 was highly enriched in both the M and S complexes, with more enrichment in the S complex than the M complex. As originally described by Yamaguchi and Yoshikawa (25), the S complex was also always more enriched for *purA16* than the M complex, and the S complex was not enriched for *metB5*. The precise chemical nature of the pSL103-containing S complexes has not been determined.

Less than 5% of exogenous ^{3}H-labeled pSL103 added during lysis was found to sediment with the M and S complexes, indicating the high MEIs for pSL103 were not a result of trapping of free plasmid DNA in the M and S complexes. The high sedimentation rate of the M complex (greater than 400S) and the S complex (100 to 150S), and the fact that purified plasmid DNA does not cosediment with either complex, make it unlikely that the enrichment of pSL103 in either complex was due to special sedimentation properties of plasmid DNA.

Approximately 60 to 90% of the total Nm^r transforming activity was found in either the M fraction (Table 1) or the combined M and S complexes (Table 3). Since the transforming activity is directly proportional to the amount of plasmid, the majority of pSL103 molecules are

not found free in the cell, but rather are associated with rapidly sedimenting, membranous particles. Greater than 50% of the pSL103 molecules remained in the M and S complexes after recentrifugation of the complexes in sucrose gradients (Table 3). Thirty-six percent of the plasmid also remained associated with the M fraction after recentrifugation in CsCl-sucrose gradients (Table 1). The fact that pSL103 sediments in the M fraction, M complex, and S complex after recentrifugation indicates stable plasmid-membrane

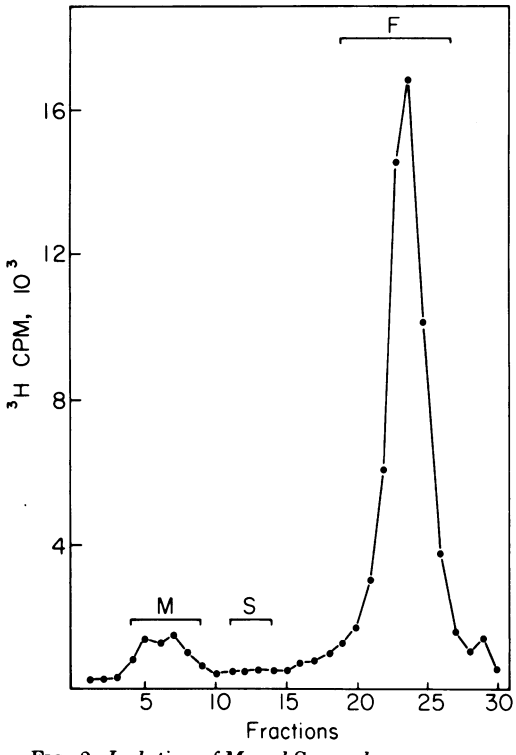

FIG. 2. *Isolation of M and S complexes on sucrose density gradients. B. subtilis 168 trp thy containing pSL103 was grown in 25 ml of SPC$^+$ (1) at 37°C (168 dna-1 was grown at 32°C) supplemented with 5 μg of thymine per ml and containing 5 μCi of [^{3}H]thymine per ml and 5 μg of neomycin per ml. At Klett 40, cells were harvested and frozen. After thawing, cells were resuspended in 0.5 ml of TKE (20 mM Tris·hydrochloride [pH 8.1], 0.1 M KCl, and 1 mM EDTA), 0.1 ml of 5 mg lysozyme per ml, and 0.05 ml of 0.1 M 2-mercaptoethanol. After 15 min at 37°C, 0.1 ml of 5% Brij-58 was added. The samples were sheared and centrifuged as described previously (25), except the gradients were centrifuged for 3 h at 20,000 rpm. The gradients were processed, transformations were performed, and MEIs were calculated, as described in the legend to Table 1. For the recentrifugation experiments, M and S complexes were dialyzed against TKE and then centrifuged under identical conditions to the first centrifugation. Direction of sedimentation is right to left.*

TABLE 3. *MEIs calculated from DNA-membrane complexes isolated from sucrose density gradients*[a]

B. subtilis strain	MEI			% pS1103[b]
	Nm^r	*purA16*	*metB5*	
168 *trp thy*				
M complex	5.2	4.4	1.3	
S complex	12.5	13.5	0.7	
dna-1 trp thy, Expt 1				
M complex	18.1	3.5	1.2	53
S complex	20.3	16.9	0.7	30
dna-1 trp thy, Expt 2				
M complex	8.4	6.3	1.2	75
S complex	11.8	10.9	0.2	12
Recentrifugation, Expt 1				
M complex				58
S complex				51

[a] M and S complexes were isolated as described in the legend to Fig. 1, and MEIs were calculated as before (18).

[b] The percent pSL103 was calculated from the total percentage of Nm^r transformants in the M or S complex.

complexes exist and further supports the notion that plasmid DNA is associated with membrane and with S complex-type particles in vivo.

In conclusion, these results have demonstrated that *S. aureus* plasmids in *B. subtilis* are preferentially associated with the cell membrane and an S-type complex. Further experiments with initiation mutants have indicated the membrane association of the plasmid plays a critical role in the initiation and replication of the plasmid (S. Winston and N. Sueoka, Proc. Natl. Acad. Sci., in press).

We thank P. Lovett for sending plasmid-containing strains and J. Beeson for critical reading of the manuscript.

This work was supported by National Science Foundation grants PCM76-20610 A01 and PCM78-11170 to N.S. and funds from Public Health Service training grant T32 On-7135 from the National Institutes of Health.

LITERATURE CITED

1. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741-746.
2. **Beeson, J., and N. Sueoka.** 1979. Membrane enrichment of genetic markers close to the origin and terminus during the DNA replication cycle in *Bacillus subtilis*. J. Bacteriol. **139**:911-916.
3. **Biessman, H., E. A. Craig, and B. J. McCarthy.** 1979. Rapid quantitation of individual RNA species in a complex population. Nucleic Acids Res. **7**:981-996.
4. **Bott, K. F., and G. A. Wilson.** 1967. Development of competence in the *Bacillus subtilis* transformation system. J. Bacteriol. **94**:563-570.
5. **Canosi, U., G. Morelli, and T. Trautner.** 1978. The relationship between molecular structure and transfor-

mation efficiency of some *S. aureus* plasmids isolated from *B. subtilis*. Mol. Gen. Genet. **166**:259–267.

6. **Denhardt, D.** 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–646.
7. **Ehrlich, S. D.** 1977. Replication and expression of plasmids from *Staphylococcus aureus* in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **74**:1680–1682.
8. **Ganesan, A. T., and J. Lederberg.** 1965. A cell membrane bound fraction of bacterial DNA. Biochem. Biophys. Res. Commun. **18**:824–834.
9. **Helling, R. B., H. W. Goodman, and H. W. Boyer.** 1974. Analysis of endonuclease R-*Eco*RI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. J. Virol. **14**:1235–1244.
10. **Imada, S., L. Carrol, and N. Sueoka.** 1976. DNA-membrane complex in *Bacillus subtilis*, p. 116–122. *In* D. Schlessinger (ed.), Microbiology—1976. American Society for Microbiology, Washington, D.C.
11. **Ivarie, R. D., and J. J. Pene.** 1970. Association of the *Bacillus subtilis* chromosome with the cell membrane: resolution of free and bound deoxyribonucleic acid on renografin gradients. J. Bacteriol. **104**:839–850.
12. **Keggins, K. M., P. Lovett, and E. V. Duvall.** 1978. Molecular cloning of genetically active fragments in *Bacillus* DNA in *Bacillus subtilis* and properties of the vector plasmid pUB110. Proc. Natl. Acad. Sci. U.S.A. **75**:1423–1427.
13. **Lis, J., L. Prestridge, and D. S. Hogness.** 1978. A novel arrangement of tandemly repeated genes at a major heat shock site in *D. melanogaster*. Cell **14**:901–919.
14. **Rigby, P. W. V., M. Dieckmann, L. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
15. **Shivakumar, A. G., and D. Dubnau.** 1978. Plasmid replication in *dna* TS mutants of *B. subtilis*. Plasmid **1**:405–416.
16. **Snyder, R. W., and F. E. Young.** 1969. Association between the chromosome and cytoplasmic membrane in *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **35**:354–362.
17. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
18. **Sueoka, N., and W. G. Quinn.** 1968. Membrane attachment of the chromosome replication origin in *Bacillus subtilis*. Cold Spring Harbor Symp. Quant. Biol. **33**: 695–705.
19. **Sueoka, N., and J. M. Hammers.** 1974. Isolation of DNA-membrane complex in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **71**:4787–4791.
20. **White, K., and N. Sueoka.** 1973. Temperature sensitive DNA synthesis mutants of *Bacillus subtilis*. Appendix: theory of density transfer for symmetric chromosome replication. Genetics **73**:185–214.
21. **Winston, S., and T. Matsushita.** 1975. Permanent loss of chromosome initiation in toluene-treated *Bacillus subtilis* cells. J. Bacteriol. **123**:921–927.
22. **Winston, S., and T. Matsushita.** 1976. Protein synthesis and release of the replication terminus from the cell membrane in *Bacillus subtilis*, p. 123–127. *In* D. Schlessinger (ed.), Microbiology—1976. American Society for Microbiology, Washington, D.C.
23. **Yamaguchi, K., S. Murakami, and H. Yoshikawa.** 1971. Chromosome-membrane association in *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **44**:1559–1565.
24. **Yamaguchi, K., and H. Yoshikawa.** 1975. Association of the replication terminus of the *Bacillus subtilis* chromosome to the cell membrane. J. Bacteriol. **124**: 1030–1033.
25. **Yamaguchi, K., and H. Yoshikawa.** 1977. Chromosome-membrane association in *Bacillus subtilis*. III. Isolation and characterization of a DNA-protein complex carrying replication origin marker. J. Mol. Biol. **110**:219–253.
26. **Yoshikawa, H., K. Yamaguchi, M. Seiki, N. Ogasawara, and H. Toyoda.** 1978. Organization of the replication-origin region of the *Bacillus subtilis* chromosome. Cold Spring Harbor Symp. Quant. Biol. **43**:569–576.