ASSOCIATION BETWEEN THE CHROMOSOME AND THE CYTOPLASMIC MEMBRANE IN BACILLUS SUBTILIS [1]

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Preparations of membranes of Bacillus subtilis isolated by differential centrifugation contain approximately 5%, 10% and 0.5% of the cellular DNA, protein and RNA respectively. The specific activity (transformants/μg DNA) of the membrane associated DNA (M-DNA) is 2-3 fold higher than the DNA isolated by conventional procedures. The M-DNA isolated from late logarithmic and stationary cultures is selectively enriched in markers at the origin and the terminus of the chromosome. The extent of enrichment can be increased by exogenous or endogenous nucleases. These results suggest that the origin and the terminus of the chromosome are associated with the membrane.

During the four decades following the discovery of genetic transformation by Griffith (1), a wealth of information has been accumulated concerning the structure and function of the transforming principle. The twenty-four years after the classic study of Avery, MacLeod and McCarty (2) have been punctuated by dramatic explosions of knowledge of the structure, replication, and translation of the genetic code. Recently, attention has been directed to the mechanism by which bacterial cells partition their chromosomes in an orderly fashion. Jacob (3) suggested that the chromosome might be segregated by attachment to the membrane. Morphologic (4-6) and biochemical analysis (7,8) have supported this contention. Because the order of replication (9) and genetic map (10) of the chromosome of Bacillus subtilis have been defined, we used a genetic analysis to determine if specific markers are associated with the cytoplasmic membrane. The data not only show an enrichment of markers at the origin and terminus of the chromosome but demonstrate that the specific activity of the membrane associated DNA

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(M-DNA) is greater than the DNA isolated by conventional techniques. These results confirm and extend the independent study of Sueoka and Quinn which was presented during the course of this investigation (11).

Materials and Methods

Bacterial Strains. To minimize genetic incompatibilities among recipient strains, all mutant loci were transferred to B. subtilis 168 (12). The try-2 locus serves as an internal control (Table 1). Contamination of M-DNA with spores led us to use an asporogenic mutant, BLJ1, for the isolation of DNA.

Preparation of Membrane Associated DNA. Cultures of the donor strain BLJ1 were grown for 3, 7.5 and 18 hrs in standard minimal medium (SMM), (13). In other experiments, a thymine auxotroph (14) was grown to stationary phase in SMM containing 6 μg/ml thymine and 0.063 μc/ml thymine-2-^{14}C (specific activity 26.2 μc/mM). The cells were harvested by centrifugation at 4 C, washed once in potassium phosphate buffer (pH 7.0, 10 mM) containing 150 mM NaCl, and 15 mM sodium citrate (PSC). The pellet was resuspended in minimal medium containing 500 mM sucrose, and lysozyme (200 μg/ml). After 1 hr at 37 C, an aliquot of the viscous lysate was treated with RNase, trypsin and desoxycholate and precipitated in ethanol (15) or isopropanol (16). The precipitate was resuspended in PSC (L-DNA). The remainder of the lysate was centrifuged for 100 min at 4 C at 39,000 x g. The pellet was homogenized in PSC containing 0.3 mM of EDTA (PSCE) in a loose fitting Ten Brock homogenizer and centrifuged at 39,000 x g for 20 min. After the sixth wash the membrane suspension was not viscous. In early experiments the washing was continued 10 to 15 times, however, more recently it was noted that beyond six washes the biologic activity of the preparation remained constant. Aggregated membranes and fragmented bacteria were removed by centrifugation for 10 min at 11,000 x g at 4 C. The supernatant fraction is designated M DNA. In some experiments aliquots of the M-DNA were extracted with phenol (17), treated with pronase (17), or incubated at 25 C for 5 min with 0.1% sodium dodecal sulfate (SDS). After 18 hrs at 4 C, the SDS treated preparation was centrifuged at 10,000 x g for 10 min at 4 C and precipitated in 4 volumes of ethanol. These extracts of M-DNA were stored after ethanol precipitation in 150 mM NaCl containing 15 mM sodium citrate. Only sterile preparations were used in this study.

Transformation. Competent cultures were obtained by a minor modification of the two step method (12). The auxotrophic requirements of the recipient strains were satisfied by supplementing with 50 μg/ml of the appropriate amino acid or purine. In the

experiments reported in this study the frequency of transformation varied from 0.1 to 1.3% with different auxotrophs.

Chemical assays. RNA and protein were measured as described previously (15). Because the color reaction with diphenylamine (15) developed more slowly in the presence of membranes the incubation was extended to 30 hrs. Under these conditions the content of DNA as determined by the colorimetric assay was similar to that calculated from the radioactivity of the preparation.

Results

Homogenization and centrifugation of the membrane preparation in PSCE resulted

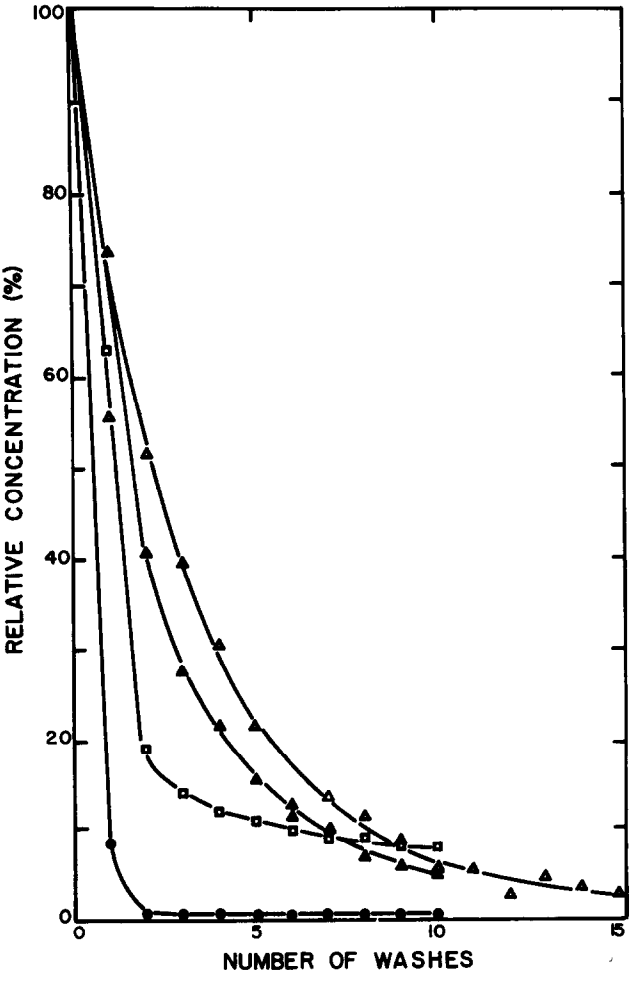

Fig. 1. Loss of DNA, RNA and protein from cytoplasmic membranes of B. subtilis during washing. RNA, ●—●, Protein, □ —□, and DNA, ▲ — ▲, washed in PSCE. DNA △ — △ in membranes washed with SSC.

in rapid removal of the RNA. After six washes less than 12% of the total cellular DNA remained associated with the membrane (Fig. 1). Further washing removed all but 5% of the DNA initially present in the preparation. Although there was some variability in the 12 preparations studied, the final DNA content ranged from 3 to 8% of the initial cellular DNA. Omission of EDTA from the buffer retarded the dissociation of the DNA (Fig. 1).

Competent cultures were incubated with various concentrations of DNA to determine the relative efficiency of the M-DNA in genetic transformation. Despite the large number of homogenizations the specific activity (transformants/μg DNA) of the M-DNA was 2 to 3 fold greater than the L-DNA (Table 2).

If the DNA was extracted from the membrane by treatment with phenol, sodium dodecal sulfate, or pronase the biologic activity remained constant for at least two months. By constrast, when crude preparations were stored at 4 C, a change in the ratio of various markers was observed. The more rapid inactivation of the biologic activity of the try-2 locus (Table 3) may be related to a membrane associated endonuclease. A relative enrichment of the ratio of Ade/Try transformants could also be obtained by treat-

TABLE 1. Summary of strains

Strain	Genotype	Source
BR19	hisA1 try-2	E. Nester
BR27	purA16 try-2	N. Sueoka
BR31	metB6 try-2	N. Sueoka
BR54	leu-7 try-2	C. Anagnostopoulos
BR62	purB6 try-2	N. Sueoka
BR64	lys-2 try-2	C. Anagnostopoulos
BR72	ura14 try-2	N. Sueoka
BR77	thr-5 try-2	N. Sueoka
BR141	metA1 try-2	C. Anagnostopoulos
BR151	lys-3 metB11 try-2	C. Anagnostopoulos
BLJ1	spoC1	M. Rogolsky

All mutants were made by introduction of the mutant loci into B. subtilis 168 by congression (12).

TABLE 2. Specific activity of M-DNA and DNA extracted from lysates

Preparations	Concentration	Transformants	Specific Activity
	γ g/ml	per ml x 10^{-2}	per ml x 10^{-5}
M-DNA	140	460	3.3
	14	37	2.6
	1.4	4.3	3.1
L-DNA	760	860	1.1
	76	97	1.3
	7.6	13	1.7

Various concentrations of M-DNA and L-DNA were incubated with competent cultures of BR27 for 30 min at 37 C with vigorous aeration. The reaction was terminated with DNase (50 µg/ml) and aliquots examined for transformation from tryptophan auxotrophy to prototrophy. Specific activity is calculated as transformants/µg DNA.

TABLE 3. Change in biologic activity of transforming DNA with storage of membranes at 4 C

Time at 4 C	Transformants	
	Ade	Try
days	per ml x 10^{-3}	
1	380	220
3	240	120
7	536	156
21	137	83
105	201	5

A preparation of M-DNA was stored at 4 C. At intervals samples were removed for assay as described in Materials and Methods.

ing the preparation with low concentrations of DNase, however, the extent of enrichment was variable and not reproducible.

The relative transforming activity for genetic markers was determined by incubating competent cells with M-DNA and L-DNA isolated from cells grown to early stationary phase. Some flucuations were noted with different preparations. To minimize this effect, the M-DNA preparation was assayed within 3-7 days or extracted with sodium dodecal sulfate within 24 hrs after the final wash. The relative marker ratio was calculated by the double ratio method of Sueoka and Yoshikawa (18) in which the number of Try transformants serves as an internal control. By this method M-DNA is normalized to L-DNA by dividing the ratio of the variable marker to reference marker (Try) of the M-DNA by that of the L-DNA. As shown in Fig. 2, there is a consistent enrichment in markers near the origin and terminus of the chromosome. The maximal difference between the purA16 marker near the origin and the markers near the mid-portion of the genetic map is approximately ten fold. The ratio of markers in the DNA extracted from crude lysates (L-DNA) was 1.0 ± 0.2 for all markers studied. Thus, the chromosome was almost in the completed state at the time of isolation of DNA (18).

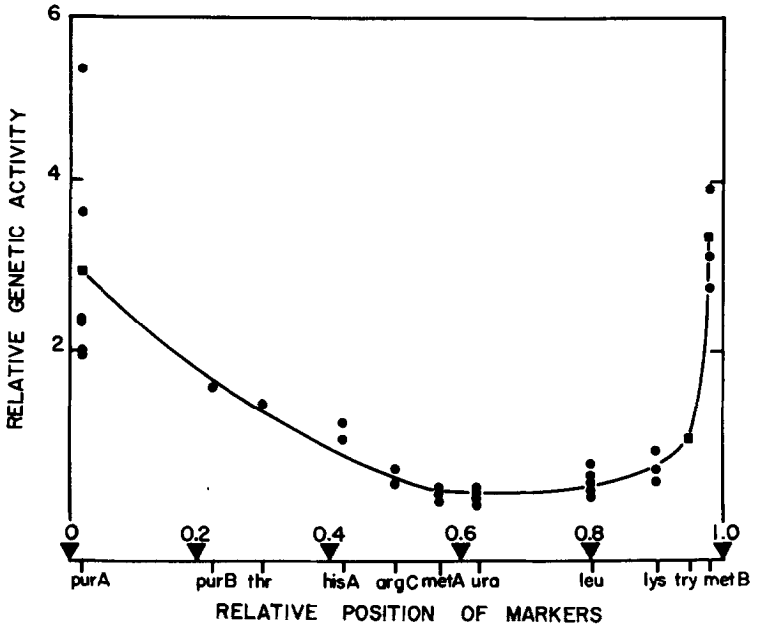

Fig. 2. Marker ratio of various loci in M-DNA isolated from stationary phase cells. The marker ratio was calculated by dividing the ratio of the variable markers to the standard marker (Try) of the M-DNA by that of the L-DNA.

TABLE 4. Biologic activity of M-DNA and L-DNA prepared from LJB1 at various stages of growth.

Preparation	Stage of growth	Transformants		Marker ratio	
		Ade/Try	Met/Try	Ade	Met
		per ml x 10^{-3}			
M23	LL	27/9	110/62	2.0	2.0
L23		202/131	91/100		
M18	ES	27/9	250/100	2.4	3.1
L18		65/51	270/340		
M20	ES	37/14	220/90	2.5	2.5
L20		420/390	950/950		
M31	LS	48/19	208/106	2.3	2.2
L31		380/350	241/268		

Competent cultures of BR27 and BR31 were incubated with M-DNA or L-DNA isolated from late logarithmic (LL), early stationary (ES) and late stationary (LS) phases of growth. The final DNA concentration during incubation with competent cells ranged from 0.1 - 1.4 µg/ml. The marker ratio was obtained by dividing the ratio of the variable marker to the standard marker (Try) of the M-DNA by that of the L-DNA.

To determine whether there was a constant enrichment in the markers at the origin and terminus M-DNA and L-DNA were prepared from cells grown for 3, 7.5 and 18 hrs in SSM. The marker ratio calculated by the double marker method for Ade and Met in M-DNA is 2.0 or greater at these periods (Table 4). Thus, there is an enrichment for the markers at the origin and terminus in all phases of growth examined to date.

Discussion

From the hypothesis of Jacob and co-workers (3), the morphologic studies, and the biochemical experiments on the association of DNA with the cytoplasmic membrane (4-8) one would predict that the genetic activity of markers adjacent to the site of chromosomal attachment would be greater in M-DNA at stationary phase or under conditions of

thymine starvation. An increased genetic activity was observed for both purA and metB. These findings indicate that the chromosome is probably attached to the membrane near the origin and the terminus. To date, a linkage between the purA and metB loci has not been established by PBS1 mediated transduction. If there were an extensive attachment of these loci to membrane, it might not be possible to obtain a single fragment of DNA containing both the purA and metB loci. Alternatively, there may be a large distance between purA and metB.

Sueoka and Quinn (11) presented data that also demonstrated a relative enrichment in the membrane fraction of markers at the origin and terminus of the chromosome. In their study, lysates of B. subtilis W23 were layered on sucrose gradients. The genetic activity of the rapidly sedimenting DNA (membrane fraction) was compared to the genetic activity of "free DNA" by transformation of B. subtilis 168. Because the DNA of the B. subtilis W23 does not transform all genetic markers at the same frequency (D. Dubnau, personal communication), the use of a heterologous system is hazardous. Nevertheless, Sueoka and Quinn observed a 1.9 fold increase in purA transformants and a 1.3 fold increase in metB transformants. In their experiments the leu locus was used as an internal standard whereas in our study the try-2 locus served as the reference. The try-2 locus is nearer the terminus of the chromosome than the leu locus. In the present study, there is a 6-fold difference between the frequency of transformation for the purA16and leu-7 loci and a 10-fold enrichment of the purA16 marker with respect to the metA and ura loci.

The data presented in Table 3 demonstrate that the rate of inactivation of the purA16 locus is slower than the try-2 locus. Enrichments as high as 60-fold were obtained with storage of M-DNA at 4 C and by treatment of M-DNA with DNase 1. Again, this was due to a more rapid inactivation of the try-2 than either the purA or the metB loci. The rate of inactivation of purA, metB and try-2 is similar in L-DNA (unpublished observations). Therefore, the increase in biologic activity of the purA and metB markers is due in part to protection of these loci from inactivation with nuclease. The enrichment of these loci during logarithmic as well as stationary phases of growth suggests that the site on the chromosome which is bound to the membrane does not vary during growth. Experiments with synchronous cultures are in progress to test this hypothesis.

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