Membrane attachment of the chromosome in *Bacillus subtilis* mutants temperature-sensitive in DNA replication

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ABSTRACT

We have examined three mutants of *Bacillus subtilis* temperature sensitive in DNA initiation and one temperature sensitive in DNA elongation, in order to investigate whether these lesions can cause or can result in a detachment of the membrane-bound chromosomal region.

Our results argue against any effect of the mutations examined on the association between the chromosome and the membrane.

INTRODUCTION

The possibility of a membrane attachment of the bacterial chromosome was first postulated by Jacob, Brenner and Cuzin¹ within the general frame of the "replicon" theory in order to explain the ordered segregation of the chromosomes during cell division. In the subsequent years a remarkable amount of evidence has accumulated to support this hypothesis. Starting with the electron-microscopic observation of Ryter and Jacob² many data have been reported which suggest a membrane binding of the replication fork in both *B. subtilis* and *E. coli* (Ganesan and Lederberg³; Smith and Hanawalt⁴; Hanawalt and Ray⁵). Membrane attachment seems also to involve the origin(s) of chromosome replication and possibly the terminus (Sueoka and Quinn⁶; Fielding and Fox⁷; O'Sullivan and Sueoka⁸). Also random chromosome fragments seem to be associated with membranous material (Rosenberg and Cavalieri⁹; Ivarie and Pène¹⁰); these last results have however been criticized on the ground that chromosome fragments might contain single-strand regions which could artifactually bind to hydrophobic regions of the membrane (Huberman, Tsai and Deich¹¹). More recently, new techniques of gentle lysis have been developed which allow the isolation of fast sedimenting structures containing the folded chromosome

and membrane material (Stonnington and Pettijohn¹²; Worcel and Burgi¹³; Worcel and Burgi¹⁴) and there is some evidence that the binding of the folded chromosome to the membrane depends on the replicative condition of the chromosome, being very weak in the case of a resting, non-replicating chromosome (Worcel and Burgi¹⁴). According to these authors the folded chromosome is released from the membrane after completion of DNA synthesis and reattaches to the membrane again, when a new cycle of DNA replication begins. This is in general agreement with the prediction of Jacob *et al.*¹ on the control of DNA replication in bacteria.

All these observations prompted us to study the DNA-membrane attachment at the non-permissive temperature in several *B. subtilis* mutants temperature sensitive in DNA replication. It is possible in fact that some of the temperature sensitive lesions can cause, or can result in, a detachment of the membrane-bound chromosomal region. Such a finding would bring further support to the hypothesis that the DNA-membrane interaction plays a central role in the regulation of chromosome replication. Such role could be exerted either at the origin on the process of initiation or in the elongation at the growing fork. We have therefore examined the DNA-membrane interaction after shift to the non-permissive temperature in three different *ts* mutants in DNA initiation and in one quick stop (elongation) *ts* mutant.

Our results argue against any effect of the *ts* mutations examined on the association between the chromosome and the membrane.

MATERIALS AND METHODS

Strains - The strains used are listed in Table I.

Media and growth conditions - In all the experiments reported here Nutrient Broth (Difco) was used, additioned with 0.5% glucose and H³-thymidine (10 µg/ml; 0.25 µCi/µg). An overnight culture was diluted 1:100 into the medium described above and grown to an A_{560nm} of 0.2 to 0.3. A portion of the culture was then shifted to the non-permissive temperature and the incubation continued for 60 min. At the end of this period the cells were collected and lysed as described below.

Fig. 1 - Sucrose step-gradient of a lysate of PB 2346 grown at 33°. Cell growth, labelling with ^3H -thymidine and lysis procedure as described in Materials and Methods. The sample was spun 90 min at 22,000 rpm in a SW 25 Spinco rotor at 4°. Fractions were collected by punching the bottom. The recovery of radioactivity was 100%.

TABLE I

Strains	Genotype	Origin
PB 2346	<u>thy</u> <u>arg</u> <u>dna-P</u>	PB 566/2 mutagenized with NTG Riva <i>et al.</i> ¹⁵
PB 2344	<u>thy</u> <u>arg</u> <u>dna</u>	PB 566/2 mutagenized with NTG
<u>dna-1</u>	<u>trp</u> <u>thy</u> <u>dna-1</u>	168 TT F. Rothman, N. Sueoka
SB 1084	<u>trp</u> <u>thy</u> <u>dna-C</u>	A. T. Ganesan

Lysis procedure - The gentle lysis procedure was that described by O'Sullivan and Sueoka⁸. 20 ml of the culture were collected, centrifuged in the cold at 10,000 x g for 10 min and resuspended in 0.3 ml of cold TESE buffer (0.05 M Tris-HCl pH 8, 0.05 M EDTA, 0.05M EGTA, 0.5M sucrose in a round bottom 15 ml centrifuge tube). Lysozyme was added (1 mg/ml final) and the cells were incubated without shaking at 37° for 60 min. At the end of this incubation more than 90% of the cells appeared as protoplasts which were subsequently lysed by adding 1.56 ml of TES buffer (0.05 M Tris-HCl pH 8, 0.1 M EDTA, 0.15 M sucrose). The lysate was then sheared by mixing with a Vortex mixer for 2 min at full speed. The lysate was diluted by addition of 1 ml of water and layered onto a step gradient of 20% sucrose, 20 ml in NET/10 buffer (Smith and Hanawalt⁴) (Tris-HCl 10⁻³ M pH 8, EDTA 10⁻³ M, NaCl 10⁻² M) over a 7 ml cushion of 62% sucrose in NET/10 and spun 90 min at 22,000 rpm in a SW 25 Spinco rotor at 4°. About 20 fractions were collected and 0.5 ml of each fraction was counted for radioactivity after TCA precipitation. Usually 80-100% of the radioactivity was recovered.

RESULTS AND DISCUSSION

When B. subtilis cells grown in Nutrient Broth and uniformly labeled with ³H-TdR are lysed and centrifuged as described in Materials and Methods, the result shown in Fig. 1 is obtained. Approximately 15% of the

Fig. 2 - Effect of shift to the non-permissive temperature on the DNA-membrane binding in two DNA initiation mutants. 20 ml of the culture were shifted to 48° and after 60 min, cells were harvested and lysed as described in Materials and Methods. Sedimentation as in Fig. 1. (A) dna-P; (B) PB 2344; (—●—) 33°, (---○---) after shift to 48° for 60 min.

radioactivity is recovered as a fast - sedimenting fraction on the 62% sucrose cushion; 70% of the radioactivity remains at the top of the gradient as low molecular weight DNA and approximately 5% of the counts are recovered at the bottom of the tube (the material at the bottom contains many unlysed cells). The remaining counts are distributed along the gradient. This distribution is similar to that reported by O'Sullivan and Sueoka⁸ with the same lysis procedure in B. subtilis and not very different from the one reported by Smith and Hanawalt⁴ in E. coli with a slightly different procedure.

On the basis of the considerations reported in the introduction we decided to study the fate of this complex following the impairment of chromosome replication in several is mutants blocked either in the initiation process or in the elongation.

It is possible, in fact, that the inactivation of some of the enzymes that control either the initiation or the elongation event can lead (as a secondary effect) to the detachment of the chromosome from the membrane, or alternatively, one can think that the impairment of chromosome replication derives directly from the detachment from the membrane. That is to say that some of the dna genes could directly control the DNA-membrane binding. In order to encompass all these possibilities, we decided to study the DNA-membrane complex in cells whose chromosome had been uniformly labeled with ³H-thymidine. Three initiation mutants have been studied: two of them have been isolated in our laboratory (one, dna-P, has been characterized in detail by Riva et al.¹⁵ and Attolini et al.¹⁶; the third, dna-1, was kindly provided by N. Sueoka (White and Sueoka¹⁷). One elongation mutant (dna-C) kindly provided by A. T. Ganesan was also examined.

The experiments were performed as described under Materials and Methods, and the results are shown in Figs. 2 and 3. It can be seen that, contrary to our expectations, there is no effect of the inactivation of these genes on the DNA-membrane complex, as detected with our technique. The fraction of radioactivity associated to the fast-sedimenting material varies from 7 to 15% in the different strains and in different experi

Fig. 3 - DNA-membrane binding after shift to the non-permissive temperature of a *ts* mutant in the initiation of DNA replication: *dna-I* (A) and of a *ts* mutant in the DNA elongation process: *dna-C* (B). (—●—) 33°, (—○—) after shift to 48° for 60 min. Experimental conditions as in Fig. 2.

ments. However, this fraction does not decrease when the initiation process or the elongation process are impaired. These results indicate that either the genes examined in this work play no role in the DNA-membrane association or that our technique is inadequate to detect relevant changes in this association.

In conclusion, we do not observe any variation in the extent of binding between membrane and DNA as a consequence of the block in DNA replication either at the initiation or at the elongation level.

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