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Cloning and mapping of the replication origin of *Escherichia coli*

(closed circular DNA/restriction mapping/sequence homology/autonomous replication)

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ABSTRACT The replication origin of *Escherichia coli* has been cloned on a nonreplicating DNA fragment coding for ampicillin resistance. This recombinant DNA, named pSY211, replicates depending on the presence of the replication origin and can be recovered as a closed circular plasmid DNA of 10.7 megadaltons (Mdal). A restriction map has been constructed. *Eco*RI cleaves pSY211 into two fragments: one is the ampicillin fragment of 4.5 Mdal and the other is a chromosomal fragment of 6 Mdal and contains the origin. The 6 Mdal *Eco*RI fragment has four *Bam*HI sites, three *Hin*dIII sites, and one *Xho* I site. A mutant of pSY211 has been isolated which is lacking two *Bam*HI fragments of the chromosomal fragment. In *recA* hosts, pSY211 is lost at a high frequency. In *recA*$^+$ hosts, pSY211 is integrated into the chromosome due to nucleotide sequence homology between pSY211 and the replication origin of the *E. coli* chromosome. The integration site has been mapped. We conclude that the replication origin is located at a site between *uncA* and *rbsK*, at about 83 min on the genetic map of *E. coli*.

Replication of the *Escherichia coli* chromosome is initiated at a unique site located in the vicinity of the *dnaA-ilv* region and proceeds bidirectionally (1–3). Various theories have been proposed concerning the mechanism of initiation of replication of the *E. coli* chromosome. However, it is generally accepted that initiation involves a step in which the replication origin interacts with a substance that specifically recognizes the nucleotide sequence of the origin (4–7). Biochemical analyses of such a specific reaction in the initiation event have been hindered by the large bulk of chromosomal DNA compared to the specific site of the replication origin. It is evident that purification of the origin DNA is essential to these analyses.

Progress of research on *in vitro* recombination has made it possible to clone replication regions of F and R factors (8, 9). This was accomplished by selecting recombinant plasmids comprised of a nonreplicating DNA fragment carrying a gene determining antibiotic resistance and an *Eco*RI fragment of F or R factor.

In this paper we describe cloning of the replication origin of the *E. coli* chromosome based on the same principle as that used for F and R factors. A restriction map of the origin-containing DNA was constructed. Moreover, we have mapped the site of the replication origin at about 83 min on the genetic map of *E. coli* (10).

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. All strains used were derivatives of *E. coli* K-12. C600 (pSC138) was obtained from S. N. Cohen (8); 1100 (F$^-$ *endA thi*), from M. Sekiguchi (11); and AN120 (*argE*3 *thi*-1 *uncA*401 *strA*), from Y. Anraku (12). The following strains were from our laboratory stock: LC248 (HfrKL16 *recA*); LC256 (*ilv leu thyA*); CRT46*spc*: (*dnaA* T46 *ilv thr leu thi thyA mal lacY spc strA*); CRT4621 (HfrP4 × 8 *thi thyA phoS tnaA*). E110 (*argE proA leu thr his thi mtl xyl ara lacY galK strA tsx glmS*); JE6063 (*ara lacY gal xyl strA recA*); JE6087 [Δ(*pro-lac*) *thi*); JE6112 (*asn thi strA recA*); JE6196 (*pyrE argG thyA rbsK strA*).

Bacteria were grown in L broth (13). Ampicillin (Ap) was added to a concentration of 20 μg/ml.

Enzymes. *Eco*RI, *Bam*HI, *Hin*dIII, and T4 DNA ligase were obtained from Miles Laboratories. *Xho* I was purified from *Xanthomonas holcicola* by a procedure of T. R. Gingeras, P. A. Myers, J. A. Olsen, F. A. Hanberg, and R. J. Roberts (personal communication). *Hae* II was a gift from M. Takanami.

Conditions for the ligase reaction were as described (14).

Agarose Gel Electrophoresis. DNA samples were electrophoresed in a column (0.7 × 9 cm) of 0.7% agarose in Tris/EDTA/borate buffer (15) at 100 V for 1.5 hr at 25°. Molecular weight of DNA fragments was determined from mobility of the fragments relative to that of reference DNAs included in the sample as internal markers (16). Reference DNAs used were *Eco*RI-digested pCR1, 8.7 Mdal (17); pSF2124, 7.6 Mdal (18); ColEl, 4.2 Mdal (19); pVH51, 2.1 Mdal (20), and *Hae* II-digested ColEl, 1.6, 1.2, 0.76, 0.46, 0.23, and 0.05 Mdal (21). Uncertainty in molecular weight was estimated to be about ±5%. Fragments smaller than 0.2 Mdal were not detected in our procedure.

Preparation of Ap Fragment. As a source of Ap fragment, a ColEl–Ap plasmid, named pYT10, was constructed from *Eco*RI treatment and ligation of a mixture of pVH51 and pSC138 (Y. Takeda and S. Yasuda, unpublished data). pVH51 is a mini-ColEl plasmid (20) and pSC138 is a plasmid composed of the replication region of F factor and the Ap fragment derived from a penicillinase plasmid of *Staphylococcus aureus* (8). The *Eco*RI-digested pYT10 generated a pVH51 fragment of 2.1 Mdal and an Ap fragment of 4.5 Mdal. Each of the fragments was separated by agarose gel electrophoresis. The Ap fragment was extracted from agarose and purified by ethidium bromide/CsCl equilibrium centrifugation as described (9).

Preparation of Closed Circular DNA. A cleared lysate was prepared from cells harboring plasmids by an EDTA/lysozyme/sodium dodecyl sulfate procedure (22). After phenol treatment and removal of 2.5 M NaCl-insoluble materials, samples were subjected to ethidium bromide/CsCl equilibrium centrifugation (23). The closed circular DNA band was removed, treated with isopropanol to remove ethidium bromide, and dialyzed against 10 mM Tris·HCl/1 mM EDTA, pH 7.5.

Transformation. Cells were treated with $CaCl_2$ and transformed with purified plasmid DNA or a cleared lysate of plasmid-carrying bacteria as described (24).

Pl Transduction. Plkc was used and the procedures were as described (13).

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Abbreviation: Ap, ampicillin.

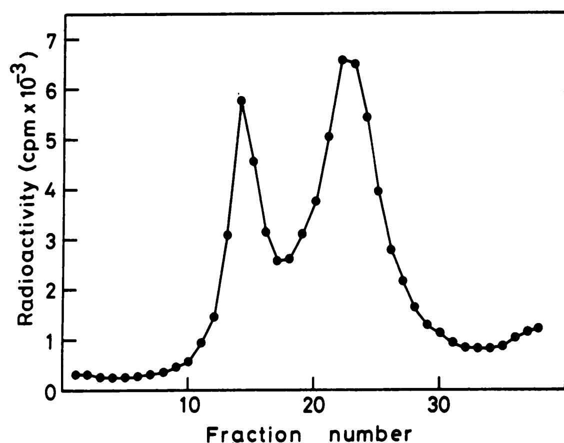

FIG. 1. Closed circular DNA of pSY211 as shown by ethidium bromide/CsCl equilibrium centrifugation. LC248 *thy*$^-$ (pSY211) was labeled in L broth containing Ap at 20 μg/ml and [^{3}H]thymidine at 1 μCi/ml. A cleared lysate was prepared and dialyzed against 0.1% Sarkosyl in 10 mM Tris-HCl/1 mM EDTA, pH 7.5. The sample was subjected to ethidium bromide/CsCl equilibrium centrifugation and radioactivity of the fractions was measured in a liquid scintillation counter.

Preparation of *E. coli* DNA. *E. coli* DNA was prepared from strain 1100 by the procedure of Cosloy and Oishi (25).

Electron Microscopy. The plasmid DNA was prepared for electron microscopy by the basic-protein film technique of Kleinschmidt and Zahn (26) with some modifications (27).

Containment. These experiments were conducted in a P2 facility.

RESULTS

Construction of Origin-Containing Plasmid. Purified Ap fragment was mixed with *Eco*RI-digest of *E. coli* 1100 DNA and treated with T4 DNA ligase. The mixture was then used to transform LC248 (Hfr *recA*$^-$), and Ap-resistant clones were selected. LC248 was used as a recipient to avoid possible contamination of F replicon–Ap recombinant plasmid. Three independent transformants were analyzed.

Cleared lysates were prepared from each of the Ap-resistant transformants and examined for the presence of closed circular plasmid DNA by ethidium bromide/CsCl equilibrium centrifugation. All of three clones LC248 (pSY201), LC248 (pSY202), and LC248 (pSY211) harbored closed circular DNA of identical size (about 10 Mdal). Fig. 1 illustrates the results of centrifugation of a cleared lysate of [^{3}H]thymidine-labeled LC248*thy*$^-$ (pSY211) cells. The left peak represents the closed circular pSY211 DNA. In a separate experiment, the copy number of pSY211 was examined by using an EDTA/lysozyme/Sarkosyl lysate (28) and was found to be about 0.8 per genome of *E. coli* (data not shown). Fig. 2 is an electron micrograph of purified pSY211 DNA. Measurement of length of 49 molecules gave a value of 10.7 ± 0.3 Mdal for pSY211.

Upon treatment with *Eco*RI, all of pSY201, pSY202, and pSY211 DNAs were cleaved into two fragments; one of 4.5 Mdal, the Ap fragment, and the other of 6 Mdal. Thus, these DNAs seem to be identical in composition. Therefore, one plasmid, pSY211, was analyzed further.

Because the Ap fragment cannot replicate by itself (8), the 6 Mdal *Eco*RI fragment must have replication function, most possibly the replication origin of the *E. coli* chromosome.

It has been established that the replication origin is placed at the *dnaA-ilv* region of the genetic map of *E. coli*. Therefore, the question of whether pSY211 carries *E. coli* genes located

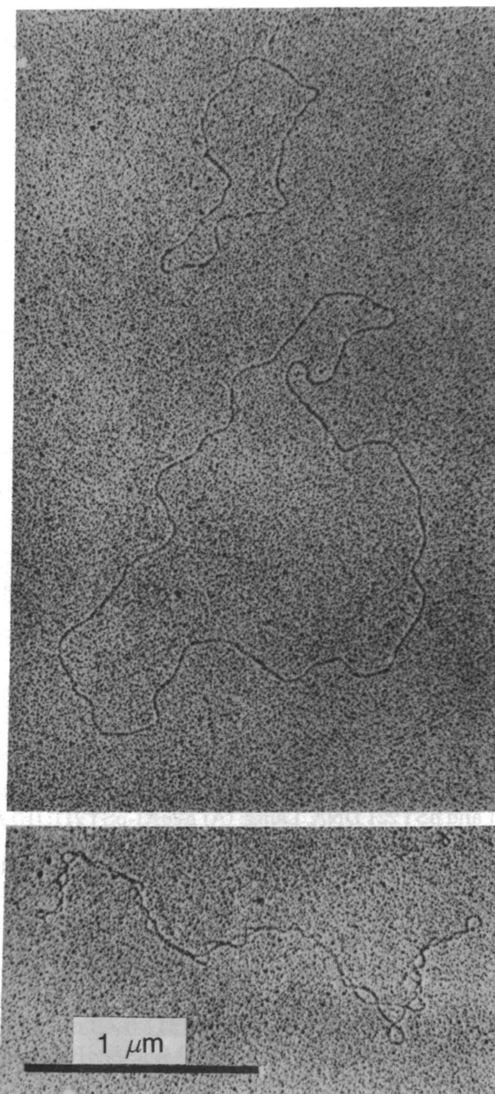

FIG. 2. Electron micrographs of three pSY211 DNA molecules. (*Upper*) Open circular ColE1 DNA at top, added as a reference, and open circular pSY211 DNA. (*Lower*) Closed circular pSY211 DNA. (Bar represents 1μm.)

at that region, including *pyrE, dnaA, asn, tnaA, phoS, uncA, rbsK,* and *ilv*, was examined. pSY211 complemented none of these markers.

Restriction Analysis of pSY211. pSY211 was treated with various restriction endonuleases and the digests were analyzed by agarose gel electrophoresis. As shown in Fig. 3, *Eco*RI cleaved pSY211 into two fragments of 4.5 and 6 Mdal. *Hin*dIII generated four fragments, of 4.2, 3.9, 1.3, and 0.8 Mdal. *Xho* I cleaved pSY211 into two fragments, of 8.0 and 2.0 Mdal. *Bam*HI produced four fragments, of 6.6, 2.0, 1.2, and 0.4 Mdal. A restriction map was constructed by digestion of pSY211 with various combinations of the restriction endonucleases. Fig. 4 illustrates the restriction map of the 6 Mdal *Eco*RI fragment of pSY211. This differs from restriction maps of either ColE1- or F-derived plasmids.

The *Hin*dIII-restriction map of the 6 Mdal *Eco*RI fragment agrees well with that of the origin-containing *Eco*RI fragment (8.6 kbases) of *E. coli* identified by Marsh and Worcel (29).

Further Cloning of Replication Region of pSY211. It has been shown in the previous sections that pSY211 replicates depending on the replication origin of *E. coli* which is carried

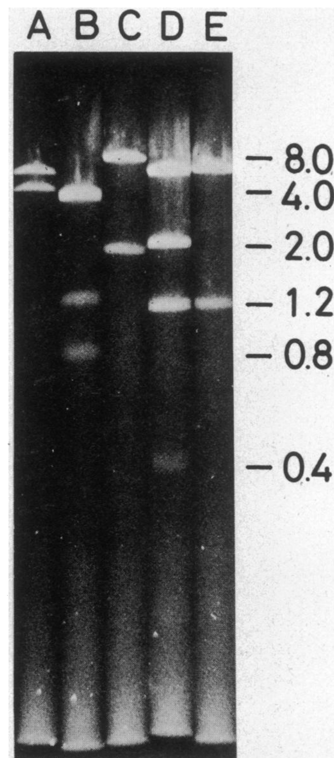

FIG. 3. Agarose gel electrophoresis of endonuclease-digested pSY211 and pSY221 DNA. Lanes: (A) *Eco*RI-pSY211; (B) *Hin*dIII-pSY211; (C) *Xho* I-pSY211; (D) *Bam*HI-pSY211; (E) *Bam*HI-pSY221. Numbers represent molecular weight in Mdal. In this electrophoresis, the largest two fragments of *Hin*dIII-digested pSY211 (lane B) were not separated completely.

in the 6 Mdal *Eco*RI fragment. In order to determine the location of the origin in that fragment, an attempt was made to isolate deletion derivatives of pSY211 by *Bam*HI digestion. *Bam*HI cleaved pSY211 only at the 6 Mdal *Eco*RI fragment and not in the Ap fragment. Thus, it was expected that, after *Bam*HI treatment, a smaller Ap-resistance plasmid would be obtained that had lost dispensable *Bam*HI fragments.

After ligation of *Bam*HI-digested pSY211 DNA and transformation, plasmid DNAs were prepared from four Ap-resistant

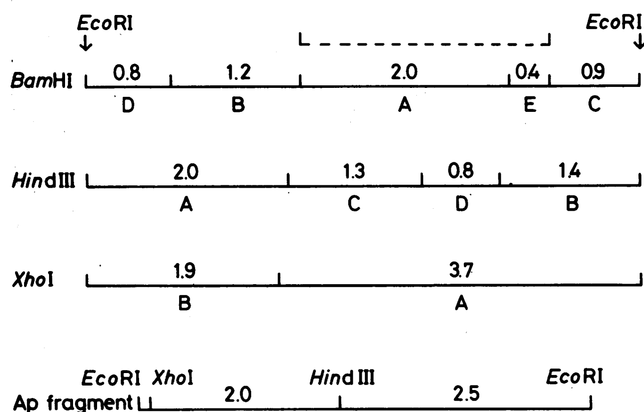

FIG. 4. Restriction map of the *Eco*RI fragment containing the replication origin of *E. coli*. Fragments are named alphabetically in order of decreasing size. Numbers represent mass of each fragment in Mdal. The broken line shows the region deleted in pSY221. Restriction map of the Ap fragment is shown in the bottom line. The maps are oriented so that one *Eco*RI site of pSY211 is assigned to the left and the other to the right.

Table 1. Cotransduction of integrated pSY211 with *dnaA* and *ilv*

Donor*	Selected marker	Unselected marker	Cotransduction frequency†	Distance, Mdal‡
JE6087	Ilv$^+$	DnaA$^+$	13/100 (13)	26.7
	DnaA$^+$	Ilv$^+$	10/100 (10)	29.0
JE6087Ap	Ilv$^+$	DnaA$^+$	10/304 (3)	37.3
		Ap^r	196/304 (64)	7.5
	DnaA$^+$	Ilv$^+$	8/373 (2)	39.4
		Ap^r	92/373 (25)	20.0

Recipient was CRT46 *spc*.

* JE6087Ap is a stable Ap-resistant derivative of JE6087 (pSY211).

† Numbers in parentheses are percentages.

‡ Distance is expressed in Mdal of DNA segments between the selected and unselected markers and was calculated according to Wu's equation (31), using 2.0 min for the size of P1 and 27 Mdal for 1 min (10).

transformants. Of four plasmid DNAs tested, three produced four *Bam*HI fragments of size identical to each of the *Bam*HI fragments of pSY211. One plasmid (pSY221) was lacking *Bam*HI fragments A and E which were present in the original pSY211 (Fig. 3, lane E). The presence of pSY221 proves that *Bam*HI fragments A and E are dispensable for the autonomous replication of pSY211. The replication origin must be located on one of three *Bam*HI fragments (B, C, or D in Fig. 4).

Evidence for Sequence Homology of pSY211 with the Origin-Region of *E. coli* Chromosome. When cultured in the absence of Ap, LC248 (pSY211) segregated cells that had lost pSY211. For example, only 1/60th of cells harbored pSY211 in an overnight culture of LC248 (pSY211).

When pSY211 was introduced into a *recA*$^+$ strain (JE6087), cells appeared in which Ap resistance was maintained stably. We inferred that, in the stable Ap-resistant cells, pSY211 may be integrated into the host chromosome by homology in DNA sequence and that the integration site may be located in the vicinity of the *dnaA-ilv* region where the replication origin of *E. coli* has been mapped (1–3). To test this possibility, linkage of the Ap-resistance gene [designated *bla* hereafter, according to Novick *et al.* (30)] with *E. coli* genes around that region was examined. A stable Ap-resistant derivative, named JE6087Ap, of JE6087 (pSY211) was used as a donor in P1 transduction. Results are shown in Table 1. It is clear that *bla* (Ap-resistance gene) was cotransduced with *dnaA* as well as *ilv*. The cotransduction frequency between *bla* and *dnaA* or *ilv* was significantly lower than that of a control experiment in which the donor was JE6087. This suggests that pSY211 is inserted between *dnaA* and *ilv*. The frequencies of joint transduction of the markers were converted into molecular weights of DNA segments between the markers, according to Wu's equation (31). The distance between *dnaA* and *ilv* was about 28 Mdal in JE6087 and about 38 Mdal in JE6087Ap. The difference is about 10 Mdal, which is the mass of pSY211. Thus, it is concluded that an entire molecule of pSY211 was integrated into the host chromosome and stretched the region.

There is a possibility that pSY211 is integrated by chance at that region in the donor strain (JE6087Ap) used in the above experiment. To exclude this possibility, 10 stable Ap-resistant derivatives of independent origin were examined for the linkage between *bla* and *ilv*. It was found that the integration of pSY211 occurred at the *dnaA-ilv* region in all of the strains (data not shown).

In the next experiment, the effect of a *recA* mutation on the integration of pSY211 into the chromosome was examined.

Table 2. Transduction mapping of integration site of pSY211

Exp.*	Genotypes of transductants†				No. of transductants‡
	ilv	*rbsK*	*bla*	*uncA*	
I	1	1	1		99 (50)
	1	1	0		54 (27)
	1	0	1		17 (8)
	1	0	0		30 (15)
II	1		1	1	32 (29)
	1		1	0	35 (32)
	1		0	1	3 (3)
	1		0	0	40 (36)

Recipient was LC256 (ilv^-) and selection was Ilv^+.

* Donors in these experiments were Ap-resistant derivatives of JE6196 and AN120 in Exp. I and II, respectively. Ap resistance of these strains had been transduced from JE6087Ap, a stable Ap-resistant derivative of JE6087 (pSY211).

† The genetic markers derived from the donors are expressed by 1 and those derived from the recipient, by 0.

‡ Numbers in parentheses are percentages of the total transductants tested.

None of three *recA* strains (LC248, JE6063, and JE6112) carrying pSY211 produced stable Ap-resistant cells after many cycles of cultivation. This demonstrates that the integration is not due to illegitimate recombination (32), but that it is due to the $recA^+$-mediated recombination through homology on DNA sequence between pSY211 and the *dnaA-ilv* region.

From all of the observations described so far, it is concluded that pSY211 contains the replication origin of *E. coli* on its 6 Mdal *Eco*RI fragment and that pSY211 replicates autonomously depending on the origin.

Mapping of the Replication Origin of *E. coli*. As described in the previous section, pSY211 contains the replication origin of the *E. coli* chromosome and is integrated into the chromosome at the *danA-ilv* region. More precise mapping of the integrated plasmid will give unambigious localization of the replication origin of *E. coli* chromosome.

Ap resistance of JE6087Ap was transduced into various strains harboring mutations in genes between *dnaA* and *ilv*. The resulting Ap-resistant transductants were used as donors in the further transduction of the ilv^+ gene into a recipient, LC256 (ilv^-). Selection was made for Ilv^+, and cotransduction of *bla* (Ap resistance) and other markers was determined. Typical results are summerized in Table 2. It is demonstrated that the *bla* gene is located at a site between *uncA* and *rbsK*. This means that the replication origin lies at that site (Fig. 5).

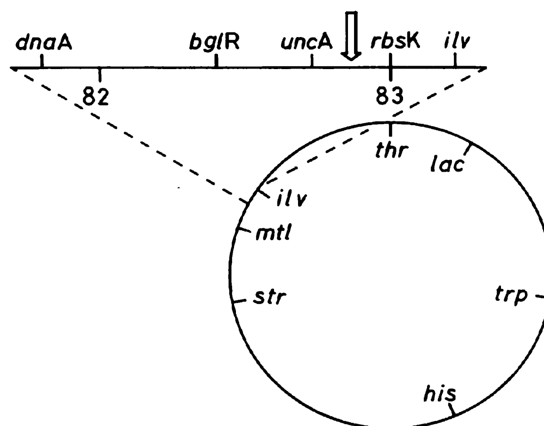

FIG. 5. The region of the replication origin on *E. coli* genetic map (10). The arrow indicates the replication origin.

DISCUSSION

It is concluded that the 6 Mdal *Eco*RI fragment of pSY211 contains the replication origin of *E. coli* from the following facts. First, it has been shown that the Ap fragment cannot replicate by itself (8), and thus the 6 Mdal fragment must contain a replication origin. Second, in $recA^-$ bacteria, pSY211 was lost at a high frequency during growth. This character resembles that of F poh^+ which is assumed to have the replication origin as reported by Hiraga (33). This could indicate that the replication origin on the plasmids competes with that on the chromosome for the initiation protein(s) essential for replication. Third, in $recA^+$ cells, the entire pSY211 DNA was integrated into the chromosome.in the *dnaA-ilv* region where the replication origin has been mapped (1–3). The integration occurred through $recA^+$-dependent recombination; thus, pSY211 should contain DNA homologous to that of the *dnaA-ilv* region. pSC138, a miniF-Ap plasmid (8) is never integrated into that region in a rec^+ host. It is concluded, therefore, that the Ap fragment plays no role in the integration of pSY211 at the region. Thus, the homology must reside in the 6 Mdal *Eco*RI fragment of pSY211. Finally, the restriction map of the 6 Mdal *Eco*RI fragment of pSY211 agrees very well with the *Eco*RI-*Hin*dIII restriction map of origin-containing DNA identified by an entirely different principle (29).

Marsh and Worcel (29) reported that the replication origin is located around the *Hin*dIII fragment of 1.3 kbases, which corresponds to *Hin*dIII fragment D of the present map of pSY211. Our successful isolation of a deletion mutant (pSY221) proves that *Hin*dIII fragment D is dispensable for autonomous replication of pSY211 and that the replication origin can not be located in that region. Their conclusion was drawn from comparison of specific activities of restriction fragments of pulse-labeled *E. coli* DNA after initiating synchronized replication, and their technique could be less accurate than ours.

Taking advantage of integration of pSY211 into the *E. coli* chromosome, we mapped the replication origin at a site between *uncA* and *rbsK*. Recently, two authors have reported that the replication origin is located at regions different from ours. Masters (34) located the replication origin at *mtl-bglR* region on the assumption that the growth of cells might be inhibited by the presence of an extra copy of the replication origin on various types of F′ factors. However, it has not been ruled out that the growth inhibition by an extra copy could occur by other mechanisms. Moreover, there is a possibility that regions containing the replication origin were deleted in her F′ factors that did not show growth inhibition. Hiraga (33) concluded that the poh^+ gene, which is assumed to be the replication origin of *E. coli*, is located at a site between *dnaA* and *bglA*. This disagrees with our conclusion. We suggest that his experimental results could also be explained on the basis of our conclusion on the location of the replication origin.

Plasmid pSY211 contains a DNA fragment of only 1/500 of the total *E. coli* chromosome and is present in cells in a closed circular form; its DNA is easy to isolate in pure form. Because it contains the replication origin of *E. coli*, it could be a useful material for the structural analysis of the replication origin and for biochemical studies on the mechanism of replication and segregation of the *E. coli* chromosome.

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