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Control of Plasmid Replication in *Escherichia coli*: Correlation of the Membrane Site of DNA Replication with the Bacterial Segregation Unit

(bacteriophage λ/repressor/heat induction/superinfection)

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ABSTRACT **The fate of parental λ genomes after superinfection of homoimmune lysogenic cells was studied. The data confirm a previous observation that in the presence of the λ repressor, superinfecting λ DNA does not become associated with replication sites on the bacterial cell membrane. Under these conditions, the nonreplicating, superinfecting phage genomes do not become associated with the bacterial segregation unit. These results support the concept that the attachment of DNA to the bacterial membrane at specific sites is involved in the control of both chromosome replication and segregation, as predicted by the replicon hypothesis.**

In their elaboration of the replicon hypothesis, Jacob, Brenner, and Cuzin (1) proposed that both the replication and the segregation of the several independent replicons that may coexist in a bacterial cell were controlled through their association with a common element of the cell membrane by means of specific attachment sites. The geometric arrangement of the sites within the membrane would ensure the equipartition of chromosome replicas into daughter cells at each cell division; the common membrane element would thus constitute the bacterial unit of segregation. There has now accumulated considerable evidence, derived from chemical and morphological studies of various bacterial and phage systems, that DNA is attached to and replicates on, or in close relation to, the cell membrane (2–13).

The evidence for the existence of the bacterial segregation unit is less direct and has been largely derived from two separate experiments (14, 15) that demonstrated the co-segregation of a nonreplicating sex-factor with a previously labeled ancestral bacterial chromosome during many generations of bacterial growth. There is, as yet, no direct evidence that the segregation unit is related either to the bacterial cell membrane, or, more specifically, to "replication sites" defined by particular chemical fractionation techniques. According to the replicon hypothesis, the attachment of a replicon to its specific replication site should be a necessary condition for its cosegregation with the bacterial chromosome. In this paper, we present the results of an experiment that we devised to test this prediction by analyzing the pattern of segregation of a superinfecting λ phage in a growing population of homoimmune lysogenic bacteria.

Previous studies have demonstrated that very early in λ infection, the injected parental phage DNA becomes associated with the bacterial cell membrane, where it subsequently undergoes replication (8). Hallick, Boyce, and Echols (16) have shown that this association depends on the function of the λ regulator gene N, and is blocked directly by the λ repressor, thereby establishing the genetic specificity of the association. More recently, however, Salivar and Gardinier (17), using a different method to detect DNA–membrane association (8), reported, in contradiction to Hallick *et al.*, that parental λ DNA *does* become associated with the membrane after superinfection of a homoimmune lysogen. This discrepancy is unresolved and leaves unsettled the important question of the mechanism by which the λ repressor *directly* inhibits the replication of a superinfecting homoimmune phage (18).

We (19) described the independent segregation of the *Escherichia coli* chromosome and nonreplicating λb2 (20) in a population of abortive lysogens, an experimental system formally analogous to that of the superinfected homoimmune lysogen. This result indicated that the λ repressor prevented the association of the phage DNA with the bacterial segregation unit, but it did not exclude the possibility that the random segregation observed was due to the absence of a structural or functional phage component related to the extensive deletion in the λb2 chromosome.

The recent conflicting reports of Hallick *et al.* and of Salivar and Gardinier suggested to us that a study of superinfected lysogens would provide the opportunity to (*a*) assess by an independent method the biological significance of the DNA–membrane association defined by the different analytical techniques, and (*b*) determine whether there was any correlation between the attachment of a replicon to its replication site and its pattern of segregation.

We first attempted to resolve the question of λ DNA–membrane association by examining the fate of parental [^{3}H]λind$^-$ (21) DNA after infection of otherwise isogenic sensitive and lysogenic cells of *E. coli* C600 (22). The particular strains used were dictated by the requirements of the segregation protocol described below. The details of the experiment and the results are presented in Figs. 1*A* and 1*B*. Within 10 min after infection of the sensitive strain C600, all of the parental λind$^-$ DNA became associated with a rapidly sedimenting structure that is taken to be evidence of DNA–membrane association. In contrast, after 30 min of superinfection of the homoimmune lysogen C600(λCI857) (23), most of the parental phage DNA remains free from the rapidly sedimenting structure. These results are identical to those reported by Hallick *et al.* (16) and confirm their observation that the λ repressor prevents the association of homoimmune superinfecting phage DNA with the cell membrane. When portions of the same lysate preparations described in Fig. 1 were analyzed for DNA–membrane association by the method of Salivar and Gardinier (17), results entirely consistent with the sucrose gradient analyses were obtained. Thus, although we have not studied the Millipore procedure

(8) in detail, we cannot support the findings of Salivar and Gardinier with respect to superinfected lysogens.

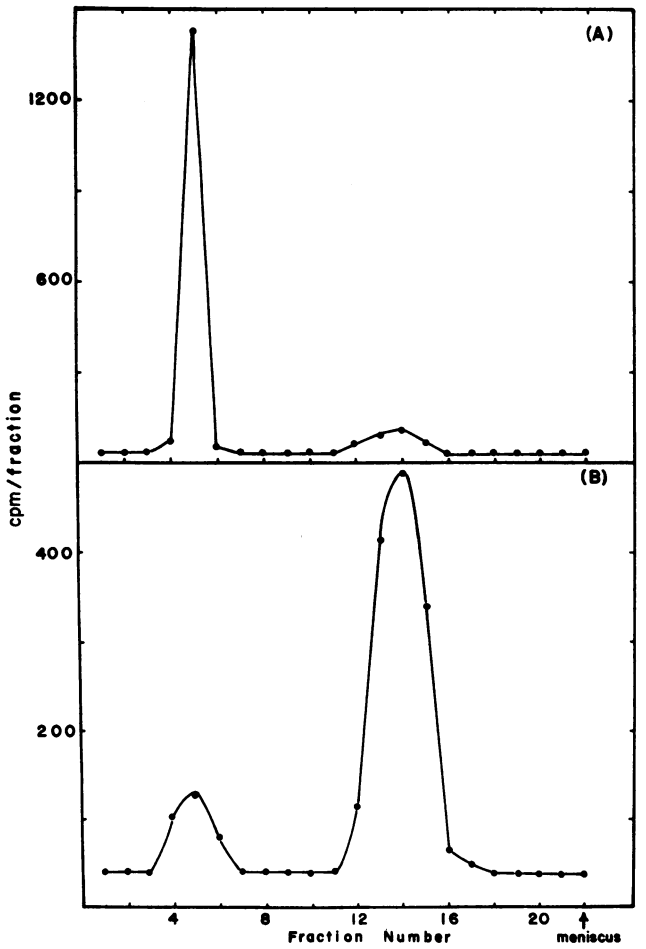

FIG. 1. Sedimentation analysis of parental λind⁻ DNA after infection of sensitive and lysogenic cells. Parallel cultures of *E. coli* C600 (22) (*A*) and C600(λCI857) (23) (*B*) were grown at 32°C to a density of 3 × 10^8 cells/ml in λ medium (24) containing 10 μg/ml thiamine, 20 μg/ml each of threonine and leucine, and 5 mg/ml of maltose. The cells were harvested, resuspended at 4 × 10^9/ml in adsorption buffer [100 of ml 0.01 M $MgSO_4$, 0.10 ml of 0.5 M $CaCl_2$, 1 ml of 50×-concentrated VBM salts (25)], and shaken for 20 min at 32°C. The starved cells were infected with 1 × 10^{10} [^{3}H]λind⁻, prepared by infection of *E. coli* CR34 (26) in the presence of [^{3}H]thymine, and purification by CsCl equilibrium centrifugation. The purified, labeled phage preparation contained 1.5 × 10^{-5} cpm/PFU. After a 20-min adsorption period at 32°C, the infected cells were centrifuged and resuspended at 3 × 10^8 cells/ml in λ medium. Adsorption of [^{3}H]λind⁻ was 99.5%. The infected C600 bacteria (*A*) were shaken at 32°C for 10 min, while the superinfected C600(λCI857) bacteria (*B*) were shaken at 32°C for 30 min. After these respective periods of incubation, the infected cells were rapidly chilled and harvested, and lysates were prepared (16). 0.2 ml of each clear lysate was layered on a 3.8-ml 5–30% sucrose gradient containing a 1-ml bottom layer of 1.7 g/ml of CsCl in 40% sucrose. The sucrose solutions were prepared in 0.05 M Tris·HCl (pH 7.4)–5 mM EDTA–0.15 M NaCl. Centrifugation was in the SW 50 rotor for 60 min at 35,000 rpm and 4°C. Fractions (about 0.23 ml) were collected from the bottom of each tube and their content of trichloroacetic acid (TCA)-precipitable radioactivity was determined by liquid scintillation spectrometry. Recovery of ^{3}H was >98% from each gradient.

We next examined the pattern of segregation of λind⁻ phage after superinfection of homoimmune lysogenic cells. The experimental strategy consisted of labeling the DNA of a thermoinducible lysogen and then superinfecting the lysogen with the homoimmune thermoresistant phage. The superinfecting phage genome, whose replication (27) and transcription (28) are inhibited by the *prophage repressor*, is nonetheless capable of producing a sufficient quantity of its own *heat-stable* repressor to prevent thermoinduction of the superinfected cells (29) (see Table 1, Exp. 2). During subsequent growth of the superinfected culture in nonradioactive medium, the nonreplicating superinfecting phage genomes are unilinearly inherited, and their distribution is compared to that of the labeled bacterial chromosomes by exposure of samples of the culture to 42°C and determination of both the fraction of total cells that can undergo thermoinduction and the fraction of the labeled bacterial DNA that is contained in those cells.

The thermoinducible lysogen selected, C600(λCI857) (23), is efficiently and irreversibly induced by a 20-min exposure to 42°C (Fig. 2*A*) and subsequently lyses. If the bacterial DNA is labeled during growth at 32°C, and the cells are then induced and incubated in nonradioactive medium containing excess DNase (19), lysis of the culture can be monitored by following the conversion of the labeled DNA to acid-soluble fragments (Fig. 2*B*).

A culture of C600(λCI857) was labeled with [^{3}H]thymidine and superinfected with λind⁻; the measured multiplicity of infection was 7:1. The superinfected cells were washed to remove unadsorbed phage and then resuspended in nonradioactive medium and incubated at 32°C (Fig. 3). At various times during the subsequent 10 generations of growth of the culture, samples were heat-challenged by a 20-min exposure to 42°C, and the fraction of the total cell population

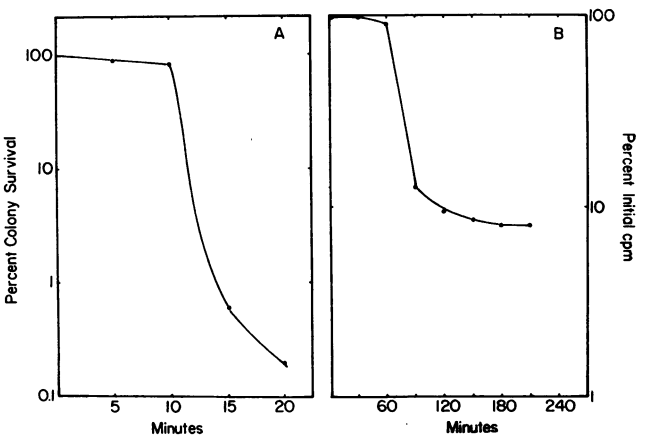

FIG. 2. Kinetics of heat induction and lysis of C600(λCI857). The thermoinducible lysogen, C600(λCI857) was grown at 32°C to about 3 × 10^8 cells/ml in λ medium supplemented with 0.1 mM deoxyadenosine (30) and 3 μCi/ml of [^{3}H]thymidine (1.9 Ci/mmol). The labeled cells were harvested, resuspended in induction medium (19), and shaken vigorously at 42°C.

(*A*). Samples were removed from the 42°C culture every 5 min and plated at 32°C for determination of colony survival.

(*B*). At the times indicated, samples were removed from the 42°C culture and assayed for TCA-precipitable radioactivity (19). An identical "lysis" curve is obtained if the labeled cells are *heat-pulsed* by incubation at 42°C for 20 min and then returned to 32°C for the duration of the experiment.

TABLE 1. *Demonstration of the presence of λind⁻ genome in thermostable segregants of superinfected lysogenic cells*

Experiment number	Percentage of thermostable cells	Percentage of thermostable cells that yield a λi[434]Sus⁺ recombinant
1.* (Control, λind⁻ MOI = 7)	100	6.5
2.† (Control, λind⁻ MOI = 1)	66	1.7
3.‡ (Segregation experiment)	100	5.5
	50	1.9
	35	1.4
	20	1.8
	6	2.0

* Exponentially growing cells of *E. coli* 594(λCI857SusA11) were starved and superinfected with λind⁻, as described in the legend of Fig. 3. The measured multiplicity of adsorption was 7:1. The superinfected cells were resuspended in induction medium containing 0.2% maltose, but without DNase, shaken at 32°C for 10 min, and then incubated at 42°C for 20 min. At the end of the heat pulse, the cells were harvested, resuspended in adsorption buffer, and again starved for 20 min at 32°C. Samples were removed for determination of total colony formers immediately before and after the heat pulse, and at the end of the second starvation period. The starved cells were then exposed for 20 min to λi[434]SusA11. Adsorption was >98%, and the measured multiplicity of infection was 1.3:1. The cells were centrifuged, resuspended in fresh adsorption buffer, and immediately plated at 32°C on a lawn of 594(λCI857SusA11) for determination of infectious centers (I.C.). The number of I.C. divided by the number of cells that had received at least one λi[434]SusA11 particle [corrected for P(0) by the Poisson equation] × 100 gives the percentage of superinfected thermostable cells that yield at least one λi[434]Sus⁺ recombinant and thus produce a plaque on the indicator lawn. No correction has been applied for the fraction of λi[434]SusA11-infected cells that may have been lost during final centrifugation.

† *E. coli* 594(λCI857SusA11) was superinfected with λind⁻ and heat-pulsed as in (*), except that the measured multiplicity of λind⁻ adsorption was 1.1:1. The determination that 66% of the superinfected cells survived the heat pulse is in excellent agreement with the predicted P(0) of superinfection given by the Poisson equation (0.33) and indicates that a single copy of the λind⁻ genome is sufficient to prevent thermoinduction of this lysogen. Immediately after the heat pulse, the culture was returned to the 32°C shaking bath for an additional 55 min to allow the inception of lysis in those cells that had undergone thermoinduction. The cells were then centrifuged, resuspended at 32°C in adsorption buffer, starved, superinfected with λi[434]SusA11, and plated for I.C. exactly as described in (*). The value in the table relates the number of I.C. observed to the number of thermoresistant cells present in the culture *immediately after the heat pulse*, and was derived in the following way: Let CF_a = number of colony formers measured immediately after heat pulse and CF_s = number of colony formers measured after second starvation period. Since the heat-resistant cells in the culture are allowed to grow at 32°C for 55 min after the heat pulse, and given that the doubling time of this strain under these conditions is about 40 min, one can predict that $CF_s \approx 2^{1.4}\ CF_a = 2.6\ CF_a$. The *observed* value of CF_s permits the calculation of a precise MOI for λi[434]SusA11 and the application of an appropriate correction, when necessary, for the P(0) of superinfection (this calculation assumes that lysing cells and cellular debris able to adsorb phage are removed by the centrisugation). The *difference* between the observed and predicted values of CF_s is due to loss of cells during the experimental manipulations. In deriving the percentage shown in the table, the measured CF_a is corrected for this loss factor, and for the appropriate P(0) of superinfection.

‡ The segregation experiment was performed as described in the legend to Fig. 3, except that no radioactivity was used and the induction medium contained 0.2% maltose and no DNase. 10 min after λind⁻ superinfection, and periodically during the ensuing 7 hr, samples were removed from the 32°C shake culture and heat-pulsed at 42°C for 20 min. The heated samples were then returned to the 32°C bath for an additional 55 min and subsequently superinfected with λi[434]SusA11, exactly as described in (†). Aliquots were taken for determination of total colony-formers immediately before and after the heat pulse and at the conclusion of the second starvation period. Measurement of I.C. values was as indicated in (*). The percentage values in the table were derived exactly as in (†).

that was thermoresistant was determined by plating. The entire culture was essentially resistant to the heat pulse by 10 min after superinfection (see legend, Fig. 3) and remained so during the first three generations of growth. Thereafter, the *absolute number* of thermoresistant cells remained constant, while the *percentage* of thermoresistant cells in the total population progressively declined to about 1%. The rate of loss of heat resistance from the culture (*insert*, Fig. 3) followed the first-order kinetics expected for the dilution of a nonreplicating element from a growing cell population. We assume that the lag of about three generations reflects the multiplicity of superinfection (7:1) and represents the time required for the growing superinfected cells to dilute the multiple λind⁻ genomes introduced at the start of the experiment to about one copy per cell.

At various times during the experiment, the culture growing at 32°C was sampled to determine total intracellular radioactivity (Fig. 4*A*). Portions of the culture aliquots that were challenged for 20 min at 42°C (Fig. 3) were returned to the 32°C bath and shaken for an additional period to allow completion of lysis of those cells that had been thermally induced (see legend, Fig. 2). As shown in Fig. 4*A*, with increasing time after superinfection the amount of radioactive DNA that remained acid-precipitable after heat challenge steadily decreased; in fact (Fig. 4*B*), the fraction of labeled DNA that was present in heat-resistant cells exactly equalled the fraction of the total cell population that was heat-resistant. This is the precise result to be expected if the nonreplicating λind⁻ genomes segregate *independently* of the labeled bacterial chromosomes that were present in the original population of superinfected cells. By contrast, if one or more of the λind⁻ genomes had remained associated with the ancestral bacterial chromosomes, then essentially all of the labeled bacterial DNA would have been present in thermoresistant cells throughout the course of this experiment (14, 15).

Our interpretation of the data in Fig. 4 is critically dependent upon the assumption that the thermoresistant cells that we assay are precisely those cells that have unilinearly inherited the nonreplicating λind⁻ genomes, and, thus, that these genomes remain capable of producing thermostable repressor sufficient to protect the cells in which they reside throughout the course of the segregation experiment. Although this assumption appears to us to be a reasonable one, no data in the literature unequivocally prove its validity. Thus, for example, an alternative explanation of our results could be proposed, namely, that immediately after super-

infection, the λind⁻ genomes initiate a burst of repressor synthesis and then become permanently inactivated. Under certain circumstances, the amount of repressor produced in a cell by nonmultiplying λ genomes is sufficient to be functionally detectable in daughter cells that do not contain phage for several generations (29, 31). According to this argument, the lag and subsequent kinetics of loss of thermoresistance from the culture (Fig. 3), as well as the data in Fig. 4, could all be explained by the dilution of multiple "units" of repressor synthesized only once during the initial period of λind⁻ superinfection. To resolve this question, we designed an experiment to demonstrate directly the presence of λind⁻ genomes in the thermoresistant fraction of the cell population.

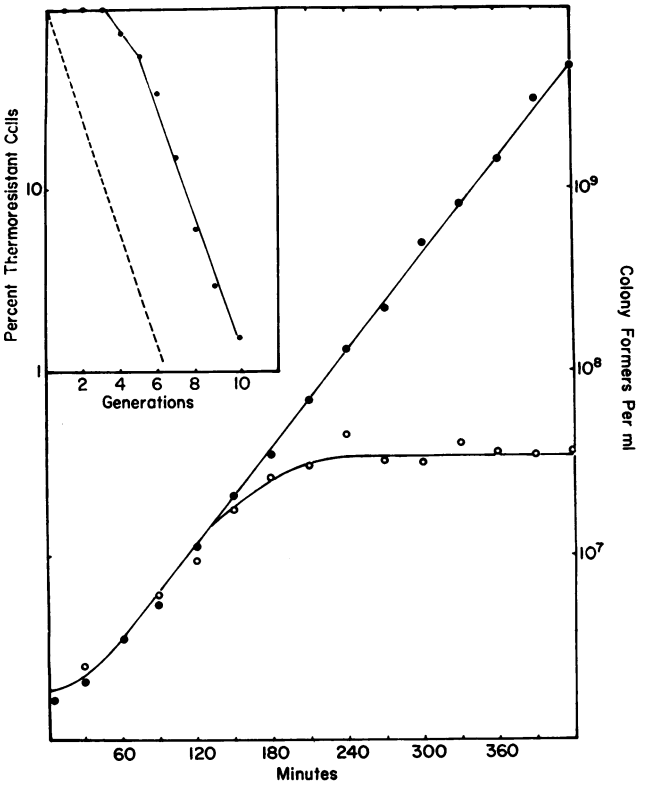

FIG. 3. Kinetics of cell growth and of λind⁻ segregation in a superinfected culture of C600(λCI857). Cells of C600(λCI857) were grown at 32°C to a density of about 3×10^8/ml in λ medium containing 0.1 mM deoxyadenosine and 500 μCi/ml of [³H]thymidine. The labeled cells were harvested, washed, and resuspended in adsorption buffer at a density of 6×10^8/ml. The cells were starved for 20 min at 32°C, and then exposed for an additional 20 min to 4×10^9 PFU/ml of λind⁻. The superinfected culture was harvested, resuspended in induction medium at 32°C at a density of 1.6×10^6 cells/ml, and vigorously shaken. 10 min after resuspension (*time zero in figure*), and every 30 min thereafter, samples were removed from the 32°C shake culture and plated at 32°C for determination of total colony formers (●—●). At the same time, an aliquot of the shake culture was transferred to a 42°C water bath, rapidly shaken for 20 min, and then plated at 32°C to assay the number of colony formers that survived the 20-min heat challenge (○—○). *The insert* plots the percentage of total colony formers in the 32°C shake culture that survive the heat pulse versus generations of bacterial growth. The *dashed line* in the *insert* predicts the rate of loss of heat resistance from the culture if thermostability is conferred upon each bacterium at time zero by a single nonreplicating λind⁻ genome that produces a single "unit" of thermostable repressor.

We first prepared the lysogen *E. coli* 594(λCI857SusA11). The host cell, 594, is nonpermissive (32), and the prophage genome contains an amber mutation in the A cistron that prevents phage DNA packaging and capsid formation (33, 34). The kinetics of heat induction of this lysogen are identical to those shown in Fig. 2*A*. The efficiency of plating of λCI857-SusA11 on 594 is $\approx 1 \times 10^{-6}$. We next constructed the phage λi^{434}SusA11, which is insensitive to the λ repressor but carries the same amber mutation as the prophage and thus cannot produce progeny on the 594(λCI857SusA11) lysogen. The efficiency of plating of this phage on strain 594 and on 594-(λCI857SusA11) is $1\text{–}3 \times 10^{-6}$. We reasoned that if the lysogen 594(λCI857SusA11) was first superinfected with λind⁻ and then with λi^{434}SusA11, recombination between the

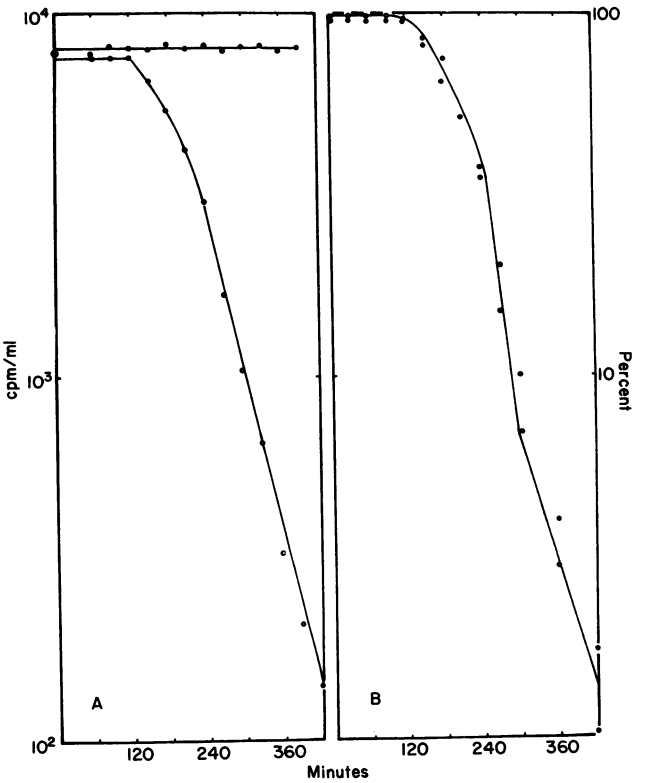

FIG. 4*A*. Thermostability of radioactive DNA in C600 (λCI857) as a function of time after superinfection with λind⁻. The data are derived from the same experiment depicted in Fig. 3. Samples were periodically removed from the labeled, λind⁻-superinfected shake culture at 32°C and assayed for TCA-precipitable radioactivity (●—●). At the same time, culture aliquots were transferred to a 42°C water bath and shaken for 20 min. These heat-pulsed samples were then returned to the 32°C water bath and incubated for an additional 160 min before assay for TCA-precipitable radioactivity (○—○). *Thus, radioactivity assay of the heat-pulsed aliquots was performed 180 min after the onset of the heat challenge (see Fig. 2B), but the data are plotted versus the time of transfer to the 42°C bath.* The points (○—○) represent radioactive DNA in cells in the 32°C culture that were still *thermostable* just before to the onset of the heat pulse.

FIG. 4*B*. Percentage of bacteria that are thermoresistant (○—○), and percentage of labeled bacterial DNA that is in thermoresistant cells (●—●). Data derived from Figs. 3 and 4*A* have been replotted to demonstrate the *random segregation* of superinfecting λind⁻ in a growing culture of C600(λCI857).

two phages could lead to the formation of a $\lambda i^{434}Sus^+$ particle that could be detected as a turbid infectious center on the indicator 594(λCI857SusA11). Such a recombination in the doubly-superinfected cell is the only event that leads with reasonable probability to the formation of a plaque on this indicator.

The experiments described in Table 1 were performed under conditions identical to those in Fig. 3. The first *control* experiment demonstrates that when the λind$^-$ superinfection is done at a multiplicity of infection (MOI) of 7:1 (as was used in the segregation experiment of Fig. 3), formation of $\lambda i^{434}Sus^+$ recombinants can be detected in 6.5% of the doubly-superinfected cells. In the second *control* experiment, in which λind$^-$ superinfection was performed at a MOI of 1:1 to simulate the conditions obtaining in the later stages of the segregation experiment, $\lambda i^{434}Sus^+$ recombination can be demonstrated in 1.5–2.0% of the heat-resistant cells. In this experiment (and in the next), the λind$^-$-superinfected cells were heat pulsed and then incubated at 32°C for an additional 55 min to permit lysis to begin in those cells that had undergone heat-induction, and to ensure that *only* thermoresistant cells would be available for λi^{434}SusA11 superinfection.

Exp. 3 is a segregation experiment performed as in Fig. 3. The data show that immediately after λind$^-$ superinfection, $\lambda i^{434}Sus^+$ recombination can be detected in 5.5% of the doubly-superinfected cells, in agreement with control Exp. 1, and that thereafter the percentage of heat-resistant cells that produce $\lambda i^{434}Sus^+$ recombinants remains *constant* at 1.5–2.0%. This constant value, identical to that observed in control Exp. 2, indicates that essentially all of the heat-resistant cells sampled throughout the experiment contain a λind$^-$ genome and, therefore, demonstrates the persistence of the λind$^-$ genome in the thermoresistant-cell fraction throughout the course of segregation.

As detailed in the legend for Exp. 3, the heat-pulsed culture aliquots were incubated for an additional 55 min at 32°C before the surviving cells were harvested for superinfection with λi^{434}SusA11. The supernatants thus obtained, which represented lysates from those cells in the sample that *had been thermally induced*, were treated with chloroform and plated on strain 594 at 42°C to determine infectious λind$^-$ progeny. In each lysate sample, the number of λind$^-$ plaques observed per heat-induced (thermosensitive) cell was $<10^{-3}$. This result is the expected corollary of the recombination data presented in Table 1 and demonstrates that λind$^-$ genomes are *not* present in the progressively expanding *thermoinducible* fraction of the growing culture.

We conclude from these data that the nonreplicating λind$^-$ genomes segregate *independently* of the labeled bacterial chromosomes present in the original population of superinfected lysogenic cells. This result is identical to that previously observed with λb2 (19) and indicates that the random segregation of λb2 in a population of abortive lysogens is not due to a structural or functional deficit that is attributable to the b2 deletion.

With respect to the conflicting reports of Hallick *et al.* (16) and Salivar and Gardinier (17), our results, obtained with two different analytical techniques, are in complete agreement with the findings of Hallick *et al.* In the presence of the λ repressor, superinfecting λ genomes do not become associated with the bacterial cell membrane at what are presumed to be specific replication sites. Prevention of this association may be the mechanism by which the λ repressor *directly* inhibits the replication of a homoimmune superinfecting phage. This mechanism may be relevant to the more-general unresolved problem of how bacterial cells control the replication of a variety of nonintegrated plasmids. We have further demonstrated that, under these conditions, superinfecting λ genomes do not become associated with the bacterial segregation unit, thus correlating DNA–membrane association with the regulation both of replication and of segregation, in support of a major prediction of the replicon hypothesis.

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