JOURNAL OF BACTERIOLOGY, Feb. 1979, p. 885-890
0021-9193/79/02-0885/06$02.00/0

Vol. 137, No. 2

Biochemical Characterization of Nonintegrated Plasmid-Folded Chromosome Complexes: Sex Factor F and the *Escherichia coli* Nucleoid

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Received for publication 20 November 1978

The existence of nonintegrated plasmid-chromosome complexes has been deduced in previous work from the cosedimentation of covalently closed, circular plasmids with host folded chromosomes. In the present work, it is shown that about 70 to 90% of the covalently closed, circular F deoxyribonucleic acid could be released in vitro from chromosome complexes by ribonuclease treatment but not by protease, Sarkosyl, or ethidium bromide. Consistent with the in vitro studies, *Escherichia coli* cells treated for 5 min with rifampin, an inhibitor of ribonucleic acid initiation, released upon lysis 90% of their plasmid deoxyribonucleic acid as freely sedimenting molecules.

Many bacteria harbor plasmids. Identification of the location of these plasmids within the bacterial cell can be useful in determining the mechanism(s) of plasmid maintenance. Only one type of cellular binding site for plasmid DNA, the cell membrane (7, 8), has been identified. Recently, we found another potential binding site, the host chromosome (11-13). This inference is based on the cosedimentation of plasmid and host chromosomal DNA which is observed in neutral sucrose gradients when care is taken to preserve the folded chromosome structure (15, 18, 21). Since the plasmid DNA is identified as covalently closed, circular (CCC) DNA, it cannot be integrated into the host chromosome.

Our preliminary data (11) indicated RNA involvement in the association of the F plasmid to the folded chromosome in *Escherichia coli*. Accordingly, we have investigated the biochemical nature of the linkage between these replicons in greater depth. The data presented here are in complete accord with our original inference that RNA links F DNA to the chromosome.

(A portion of this work was done by J.R.M. in partial fulfillment of the requirements for a doctoral degree in the Department of Biochemistry at the University of Tennessee, Knoxville.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strains CR34 and CSH50 as well as their F^+ derivatives have been described (12). Bacteria were grown at 37°C in M9 minimal medium supplemented with 0.5% (wt/vol) Casamino Acids, 300 μg of deoxyadenosine per ml, 0.2% glucose, and [^{3}H]thymidine or [^{14}C]thymine as described previously (12). Thymidine (4 μg/ml) was added to the medium when strain CR34 was used.

Isolation of folded chromosomes (nucleoids). Bacteria were lysed, and the nucleoids from the crude lysates were purified in neutral sucrose gradients by procedures previously listed (11, 12). Conditions of centrifugation were 17,000 rpm at 4°C in an SW50.1 rotor unless otherwise indicated. Procedures for fractionation of sucrose gradients, detection of radioisotopes, and computation of the percentage of plasmids cosedimenting with folded chromosomes have been described (12).

Enzymes and chemicals. Pronase (B grade) was purchased from Calbiochem and was autodigested at a concentration of 5 mg/ml in TES buffer (0.05 M Tris, 0.005 M EDTA, and 0.05 M NaCl, pH 8.0, for 3 to 5 h before use. Proteinase K was purchased from E-M Biochemicals and used without further treatment. RNase A and T_1 RNase were purchased from Calbiochem and were dissolved in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate), pH 5.0. RNase A was heated at 95°C for 10 min to destroy any contaminating DNase. Sarkosyl NL-30 was a gift from the Geigy Chemical Co.

Stability of F plasmid-folded chromosome complexes. The objective of these experiments was to characterize the biochemical basis for the plasmid-folded chromosome complexes. In general, the experimental protocol consisted of four steps. For step i, a lysate with plasmid-chromosome complexes was sedimented through a neutral, 10 to 30% sucrose gradient to purify and isolate the complexes. In step ii, the isolated complexes of step i were treated with an enzyme or detergent. In step iii, the treated complexes were resedimented through a neutral 10 to 30% sucrose gradient and fractionated into tubes, and samples of each fraction were counted. In step iv, the appropriate regions of the gradient were pooled (see Fig. 1 for an example), and the amount of CCC F DNA in each

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pool was determined by dye-CsCl centrifugation (11, 13, 17).

The release of plasmid DNA was monitored either by following the distribution of CCC and chromosomal DNA in the sucrose gradient of step iii (for example, see Fig. 1 and Table 1) or by the loss of CCC plasmid DNA from the chromosome peak, i.e., lack of cosedimentation of plasmid and chromosome. To normalize for differences in amount of material in the control and treated samples, the amount of CCC DNA in a peak or gradient region was divided by the amount of chromosomal DNA in the same region, and the ratio was multipled by 100. This normalized number is referred to as the percent CCC DNA.

RESULTS

Effect of RNases. The alkaline lability (11) of the linkage between supercoiled F DNA and a folded chromosome isolated in high salt suggests that RNA is involved in the complexing of these DNAs. Consequently, CCC F DNA should also be released from chromosomal DNA by RNase treatment. To verify this prediction, T_1 RNase was added to 1,800S plasmid-folded chromosome complexes, and they were immediately pipetted onto the top of a 10 to 30% neutral sucrose gradient. Because the chromosomes are slowly unfolded by this procedure, the generation of sheared DNA is minimal (J. Miller, unpublished observations). After incubation of the complexes for 60 min at 4°C, the treated material was sedimented to separate CCC F DNA from the bulk of the chromosomal material (Fig. 1). More than 80% of the [^{3}H]DNA sedimented with coefficients between 200 and 300S (10% sedimented to the shelf of 70% sucrose present at the bottom of the gradient). Drlica and Worcel (3) and Hecht et al. (6) have shown that an increase in angular velocity results in an apparent lowering of the sedimentation coefficient for unfolded chromosomes. Thus, because of the centrifugation conditions, the sedimentation coefficients reported here are minimal values.

The distribution of F DNA in the gradient shown in Fig. 1 was determined by measuring the amounts of CCC DNA in each of regions A, B, C, and D (Table 1). Clearly, most of the F DNA (70%, pool C) sedimented independently from the bulk of chromosomal DNA (pools A and B). Similar results were obtained when RNase A replaced T_1 RNase (data not shown). Incubation of plasmid-chromosome complexes without RNase did not release CCC F DNA, and these complexes maintained a sedimentation coefficient of 1,800S (11).

Since 90% of the DNA in pool C has the density characteristic of linear chromosomal DNA, the possibility exists that the F DNA in this pool is complexed to such fragments. If this were true, then such CCC DNA would not cosediment with added ^{14}C-labeled 80S CCC F DNA. To test this possibility, crude lysates containing ^{3}H-labeled F plasmid-folded chromosome complexes and free ^{14}C-labeled 80S F DNA were sedimented in 20 to 30% neutral sucrose gradients for an extended period after RNase treatment of the mixture. The results in Fig. 2B show that the ^{3}H-labeled F DNA cosedimented with the ^{14}C-labeled F marker and, therefore, indicate that essentially free CCC F DNA was released from complexes by RNase treatment. A comparable amount of free CCC F DNA was not observed when RNase treatment was omitted (11; Fig. 2A).

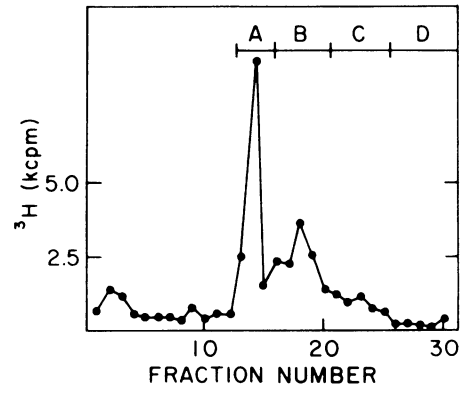

FIG. 1. *Sedimentation of T_1 RNase-treated plasmid-folded chromosome complexes. CSH50 (F$^+$), cultured with [^{3}H]thymidine (20 μCi/ml) for four generations, was harvested and lysed as described previously (12). Complexes were purified by sedimenting crude lysates through a 10 to 30% neutral sucrose gradient (data not shown). Folded chromosomes (1,600–1,900S) were then pooled, diluted with buffer to 0.4 M NaCl, and treated with 250 U of T_1 RNase in a total volume of 0.5 ml for 60 min at 4°C. Next, the treated complexes were centrifuged for 200 min at 17,000 rpm through a 10 to 30% neutral sucrose gradient containing a 70% sucrose cushion. Sedimentation in this and all subsequent sucrose gradients is from right to left. Fractions (0.18 ml) were taken by puncturing a hole in the bottom of the tube, and 20-μl samples were counted to determine radioactivity. Four pools (A–D) were then analyzed for CCC F DNA by ethidium bromide (EtBr)-CsCl centrifugation. Recovery of applied counts per minute was greater than 95% for the neutral sucrose and EtBr-CsCl gradients in this experiment and in the experiments presented in all subsequent figures. The results of the EtBr-CsCl analyses are given in Table 1.*

Effect of deproteinizing agents. Although the folded structure of the chromosome resists destruction by deproteinizing agents such as Pronase or the nonionic detergent Sarkosyl (22), the association of F plasmid with the chromosome may be labile to such agents. Complexes were incubated with or without Sarkosyl, and

TABLE 1. *Release of CCC F DNA by T_1 RNase treatment*

Pool[a]	CCC F		Chromosome	
	cpm	Distribution (%)	cpm	Distribution (%)
A	127	5	40,806	35
B	481	19	53,631	46
C	1,775	70	15,156	13
D	152	6	6,995	6

[a] The regions of the gradients in Fig. 1 labeled A through D were pooled and centrifuged to equilibrium in dye-CsCl gradients to separate CCC DNA from linear and open circular DNA. The total counts per minute found in each peak were corrected for base line contamination.

after incubation, folded chromosomes were resedimented through sucrose gradients as shown in Fig. 3. From a comparison of the percent CCC F DNA in the treated (1,660S) and untreated (1,740S) nucleoids, we conclude that less than 10% of the CCC F DNA was released by exposure to the detergent. Consistent with this conclusion, the data also show no enrichment of CCC F DNA in the slowly sedimenting material of the gradient containing treated nucleoids. Treatment of complexes with Pronase (1 mg/ml) or proteinase K (200 μg/ml) never resulted in a significant release of plasmid (eight determinations). The results with proteolytic agents were the same regardless of concentration of enzyme, salt concentration in the reaction mixtures (tests were not made below 0.4 M NaCl because nucleoids spontaneously unfold), incubation temperature (0 to 25°C), or conditions of lysis (for example, see 12). There is one exception; treatment of F plasmid-chromosome complexes with sodium dodecyl sulfate did release CCC F DNA. However, this treatment also released RNA from nucleoids, and this likely accounts for an observed efficacy of plasmid release that is comparable to RNase treatment (data not shown).

Ethidium bromide induced sedimentation changes of plasmid-folded chromosome complexes. The possibility exists that entrapped CCC F plasmids are released from complexes simply because the chromosome's hydrodynamic volume expanded as the result of the unfolding induced by RNase or sodium dodecyl sulfate. To test this possibility, the hydrodynamic volume of the plasmid-nucleoid complexes was expanded by exposure to 2 μg of ethidium bromide per ml. At this concentration of ethidium bromide, supercoils are completely removed from folded chromosomes. Consequently, the chromosomes show a lower sedimentation coefficient (1,200S) because of an increased hydrodynamic volume induced without

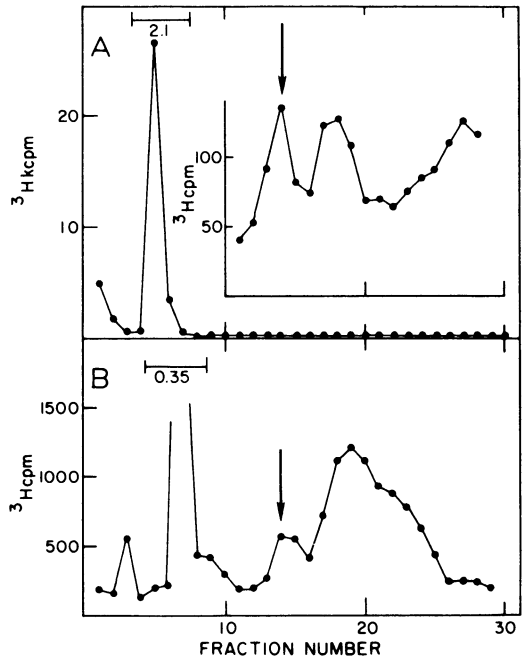

FIG. 2. *Sedimentation behavior of CCC F DNA released from RNase-treated plasmid-folded chromosome complexes. Tritium-labeled CSH50 (F^+) cells (2×10^8) were lysed in a final volume of 1 ml to release plasmid-folded chromosome complexes. Next, a 0.3-ml sample of lysate containing added ^{14}C-labeled CCC F DNA was mixed (A) with buffer or (B) with buffer containing RNase A (final concentration, 100 μg/ml). The samples were incubated at 25°C for 30 min and then slowly layered onto 4.5-ml 20 to 31% neutral sucrose gradients containing 0.5 M NaCl (10). Each gradient tube also contained a 0.5-ml cushion of 35% sucrose saturated with CsCl. After centrifugation for 100 min at 45,000 rpm and 15°C in an SW50.1 rotor, each gradient was fractionated into tubes, and 20-μl samples of each fraction were counted. In (A) fractions 11 to 30 are replotted to depict the small amount of freely sedimenting ^{3}H-labeled CCC F DNA (arrow). The fractions underneath the horizontal lines in (A) and (B) were pooled and analyzed by isopycnic centrifugation in ethidium bromide-CsCl gradients to determine the relative amount of CCC plasmid DNA in each pool. The results, expressed as percent CCC DNA, are listed under each horizontal line. The total counts per minute analyzed were: pool A, 137,000; pool B, 85,000. The conclusion that the peak in (B) at fraction 14 is CCC F DNA was verified by centrifuging the remainder of fractions 12 to 16 in a dye-CsCl gradient (data not shown).*

removal of RNA (15, 21; Fig. 4). Conditions of lysis were such that membrane-associated (peak II) and membrane-released (peak III) folded chromosomes were observable. The sedimentation rates of both chromosomal species de-

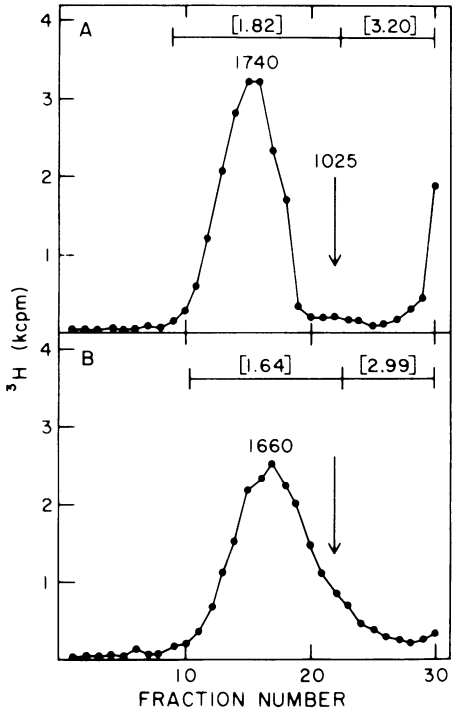

FIG. 3. *Sedimentation of Sarkosyl-treated complexes. Purified complexes from ^{3}H-labeled CSH50 (F$^+$) bacteria were incubated for 15 min at 25°C with (A) no additions or (B) in the presence of 0.5% (vol/vol) Sarkosyl. The treated or untreated complexes were then mixed with ^{14}C-labeled T4 phage and sedimented for 25 min at 17,000 rpm. Sedimentation coefficients (1,740 and 1,660S) were calculated from the peak fraction of folded chromosomes compared to the position of T4 phage (1,025S [21]). DNA material was pooled separately, as indicated by the horizontal lines, into regions representing chromosomes sedimenting with rates greater than or less than 1,000S (fraction 22). The CCC F DNA in each pool was determined by ethidium bromide-CsCl gradient centrifugation, and the measured percent CCC DNA is given above each pool. The total counts per minute analyzed in the 1,740S pool were 108,663 and represented 88% of the total counts in this gradient (A); the total counts per minute analyzed in the 1,660S pool were 101,515 and represented 80% of the total counts in this gradient (B).*

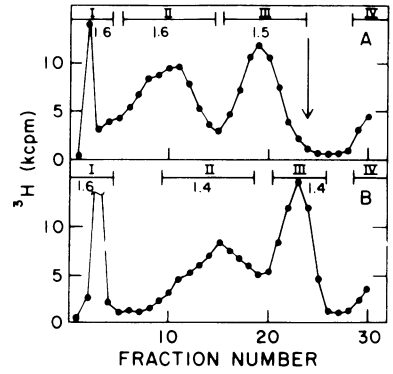

FIG. 4. *Sedimentation of complexes through neutral sucrose gradients containing ethidium bromide (EtBr). CSH50 (F$^+$) bacteria were cultured for three generations in M9 minimal medium supplemented with L-proline (20 μg/ml), harvested, and lysed. Crude lysates (0.3 ml) were mixed with ^{14}C-labeled T4 phage, layered onto 10 to 30% neutral sucrose gradients containing (A) 0 or (B) 2 μg of EtBr per ml, and centrifuged for 20 min. Gradients were fractionated as in Fig. 1, and 20-μl samples of each fraction were counted. The fractions under each horizontal bar shown in the gradient profiles were pooled and resedimented to equilibrium in EtBr-CsCl gradients to determine the percentage of CCC F DNA. The arrow in (A) represents the position of ^{14}C-labeled T4 phage (1,025S) in both gradients A and B. Fraction 3 of B contained 19,000 cpm. The numbers below each pool represent the percent CCC DNA. The total counts per minute detected in each pool were: (A) I, 320,000; II, 602,000; III, 558,000; and IV, 117,000; (B) I, 481,000; II, 561,000; III, 620,000; and IV, 100,000. The percent CCC DNA in pool (A) IV is 7.0 and that in pool (B) IV is 7.5.*

creased approximately 35% in 2 μg of ethidium bromide per ml (Fig. 4); however, the percentage of CCC DNA present in the reduced forms was essentially unchanged. Identical results were obtained when this experiment was repeated with R6K and Clo DF13 plasmid-folded chromosome complexes (M. Wlodarczyk, J. R. Miller, and B. C. Kline, unpublished data). These plasmids are much smaller than F and have masses of 26 and 6 mega daltons, respectively, Hence, the size of the plasmid in this type of analysis does not seem to be a significant factor.

Lability of plasmid-folded chromosome complexes in vivo. Consistent with the observations of others (15), lysates of rifampin-treated cells were found by us to contain essentially unfolded chromosomes (Fig. 5). Material isolated from pool A had a sedimentation rate greater than 150S. Eighty-five percent of the supercoiled CCC DNA was recovered from region B (only 20% of the chromosomal DNA was found in this region). This released plasmid DNA cosedimented with authentic 80S F DNA as judged by an analysis similar to that represented in Fig. 2B (data not shown). The experiment in Fig. 5 was repeated several times and up to 90% of the CCC F DNA could be released from its association with chromosome (data not shown).

DISCUSSION

The folded chromosome of *E. coli* is a condensed molecule composed of 40 to 80 domains of supercoiling (15, 21). Each domain is stabilized

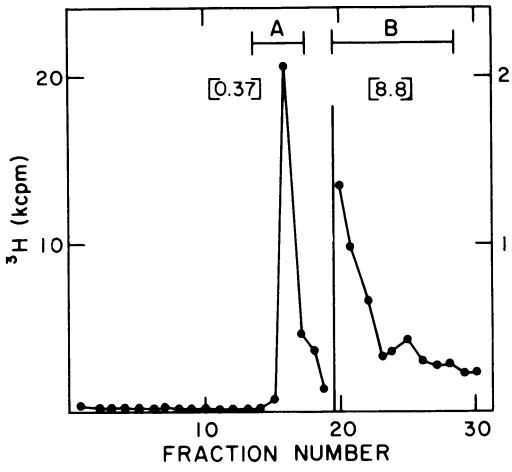

FIG. 5. *Sedimentation of plasmid and chromosomal material isolated from rifampin-treated bacteria. [^{3}H]thymidine-labeled CSH50 (F$^+$) bacteria were treated with 500 μg of rifampin per ml at 37°C for 5 min and then harvested and lysed as described in the legend to Fig. 2. A 0.3-ml sample of lysate was layered onto a 4.5-ml 10 to 30% neutral sucrose gradient formed over a 0.5-ml cushion of 70% sucrose. The gradient was centrifuged for 200 min at 17,000 rpm and 4°C in an SW50.1 rotor and then processed as described in the legend to Fig. 1. The results are plotted above. Subsequently, the fractions underneath horizontal lines A and B were pooled and analyzed in ethidium bromide-CsCl gradients. The percent CCC F DNA in each pool is given in brackets. The total counts per minute analyzed were: pool A, 89,000; pool B, 25,000.*

by nascent RNA and RNA polymerase to which the nascent RNA is bound (4, 5, 15, 21). Some additional folding may result from protein-DNA interaction, but this was only detectable after removal of RNA by RNase (3). A recent study raises questions about the validity of this last finding, and the authors contend that RNA (via RNA polymerase) is the major way of stabilizing folding in vitro (6). We found that RNA is also responsible for the in vitro association of F molecules with host cell folded chromosomes. The nature of this RNA is unknown, but the results with rifampin-treated cells suggest that it is a short-lived or nascent RNA species.

In our experiments 10 to 30% of the CCC F DNA cosedimented with the unfolded chromosomes. This may be an artifact in which released F is entrapped or aggregated to chromosomal DNA. This possibility is indicated by a reconstruction experiment which showed that this level of entrapment can occur (data not shown). Likewise, the hypersharpness of the DNA peak (Fig. 1, pool A) suggests that aggregation of DNA is occurring. We feel, however, that this hypersharpness is an artifact resulting from the large amount of highly viscous chromosomal DNA in the sucrose gradient and our technique of fractionating the gradient with a hollow needle. If this is the case, the F DNA in pool A may not have been released by RNase. Resolution of this uncertainty is very difficult because the obvious approach of using more dilute concentrations of DNA leads to great difficulty in observing the minor plasmid DNA species. Thus, we are uncertain whether RNase can release all of the CCC F DNA or only up to 70 to 90% of it. The appearance of a second chromosomal peak, the DNA in Fig. 1, pool B, was variable, and this is without consequence since we found no correlation between the size of this peak and the release of CCC F DNA from chromosomal complexes.

One would like to know how representative the CCC plasmid DNA form is for the total F population. It is generally considered that in vivo the majority of F DNA molecules are maintained in the CCC form. It is also well known that there is approximately one F per replicating chromosome and, under our growth conditions (generation time, 40 min), the ratio of plasmid to chromosome masses expressed as a percentage should be 1.82 (i.e., $[64 \times 10^6 \times 10^2/2.5 \times 10^9] \times 1/\ln 2$) (16). Thus, the fact that we observed levels of 1.4 to 1.8% CCC plasmid DNA in complexes indicates that we were examining the majority of the F population.

Archibold et al. (1) found that lowering the NaCl concentration to 0.25 M released R6K monomers from folded chromosome complexes and concomitantly decreased the sedimentation coefficient of the chromosome to 700S. R6K replicative intermediates, however, remain bound to the 700S entity but can be released subsequently by RNase or protease treatment.

Archibold and co-workers suggest that the nonreplicative R6K may be entrapped and released by the unfolding of chromosome in 0.25 M NaCl, whereas the replicative intermediates may have a more specific attachment mediated by RNA and protein. As an alternative explanation, we propose that the lower ionic strength might permit endogenous RNases to operate, and the binding of the nonreplicating R6K may be more vulnerable to such RNases than the binding of replicative molecules.

Indirect evidence also suggests that plasmids are non-integratively associated with chromosomes isolated from other bacterial species. Taichman and Rownd (19) reported that F-derived plasmids are isolated preferentially from "rapidly sedimenting material" obtained from certain *Proteus mirabilis* lysates. D. Kopecko (personal communication) also reported greater

yields of large F′ plasmids if lysates of *Proteus* species are treated with RNase before separation of plasmid from chromosome. Likewise, S. Palchaudhuri (personal communication) related that the critical factor in the recently successful isolation (14) of degradative plasmids from *Pseudomonas* species was the alkaline hydrolysis of RNA in the crude lysate. Similarly, Hansen and Olsen (4) found that alkali treatment of lysates made from *Pseudomonas*, *Salmonella*, *Escherichia*, and *Agrobacterium* species markedly enhanced recovery of most but not all plasmids.

The fact that chromosome folding is stabilized by nascent RNA which has a great tendency to aggregate in high salt concentrations (2) makes it possible that RNA stabilization of nucleoid folding is an artifact (9). Likewise, plasmid-chromosome complexing mediated by RNA may be an artifact for the same reason. However, extensive observations indicate that the frequency with which a given plasmid complexes to chromosome can be reproducibly varied to reflect events of plasmid maintenance, genetic structure, and metabolic activity (12, 13, 20). These findings do not support the possibility of artifact but neither do they completely eliminate it.

In summary, the weight of observations clearly indicates that RNA maintains plasmid-chromosome complexes, and in some cases protein may also be involved in maintenance. Complexes may form for different reasons related to replication, nonreplication, or the presence of a transposon (13). Moreover, the biochemical nature of complexes may differ from plasmid species to plasmid species. However, knowledge that plasmid-chromosome complexes do exist and are mediated by RNA in a number of bacterial genera is proving quite useful in biochemical and physiological studies of plasmid maintenance (1, 13).

ACKNOWLEDGMENTS

This work was supported in part by the Mayo Foundation and Public Health Service grants GM18608 and GM22682 awarded to B. C. K. by the National Institute of General Medical Sciences.

We thank Allen Bishop and Becky Schmitt for excellent technical assistance. We are also indebted to S. Palchaudhuri, D. Kopecko, J. B. Hansen, R. H. Olsen, E. R. Archibold, C. W. Clark, and R. J. Sheehy for sharing their findings with us before publication.

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