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Nonintegrated Plasmid-Chromosome Complexes in *Escherichia coli*

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A number of plasmid systems have been examined for the ability of their covalently closed circular deoxyribonucleic acid (CCC DNA) forms to cosediment in neutral sucrose gradients with the folded chromosomes of their respective hosts. Given that cosedimentation of CCC plasmid and chromosomal DNA represents a bound or complexed state between these replicons, our results can be expressed as follows. (i) All plasmid systems complex, on the average, at least one plasmid per chromosomal equivalent. (ii) Stringently controlled plasmids exist predominantly in the bound state, whereas the opposite is true for plasmids that exist in multiple copies or are under relaxed control of replication. (iii) The degree to which a plasmid population binds to host chromosomes appears to be a function of plasmid genotype and not of plasmid size. (iv) For the colicin E1 plasmid the absolute number of plasmids bound per folded chromosome equivalent does increase as the intracellular plasmid/chromosome ratio increases in cells starved for required amino acids or in cells treated with chloramphenicol; however, the ratio of bound to free plasmids remains constant during plasmid copy number amplification.

This paper is concerned with the intracellular location of plasmids in *Escherichia coli*. Our interest in this subject stems from the observation (17) that the majority of the autonomous, F plasmid population seems to be non-integratively complexed to its host's folded chromosomes in vivo, whereas only a small fraction of the colicin E1 (ColE1) plasmid population is complexed similarly. Aside from their size differences, the F and ColE1 systems differ in the deoxyribonucleic acid (DNA) polymerases involved in their maintenance (16, 35), in their steady-state numbers per chromosomal equivalent (3, 4, 10), in their abilities to achieve the episomal state (5), in their abilities to promote their own transmission by cell conjugation (5), and presumably in their mechanisms of plasmid segregation at cell division (9, 12). Whether or not nonintegrative plasmid-chromosome complexes are material to any of these fundamental differences is unknown.

Broadly speaking, other plasmids can be classed as mostly F-like or most ColE1-like with respect to the properties listed above; however, some plasmids, such as R6K, have some properties of both groups. The purpose in undertaking the present study was to find out if nonintegrative complexing is a general phenomenon and, if it is, to find out if the F-like and ColE1-like plasmids complex in degrees comparable to F and ColE1. The results in this article show that nonintegrative complexing is a general phenomenon and that F-like plasmids complex to a greater degree than do ColE1-like plasmids.

MATERIALS AND METHODS

Bacterial hosts and plasmids. The bacterial hosts used in these studies and the plasmids contained in them are presented in Tables 1 and 2.

Growth of bacteria. Bacteria were cultured in M9 minimal medium (17) supplemented with (final concentration): glucose, 0.5% (wt/vol); Casamino Acids (Difco, no. 0230-01), 0.5% (wt/vol); deoxyadenosine, 250 mg/ml; and, where appropriate, with thymine (2 μg/ml) or thymidine (4 μg/ml). In some cases, Casamino Acids was omitted from the medium and, instead, 20 μg of the required L-amino acids per ml was substituted.

Isolation of nonintegrated plasmid-chromosome complexes. Complexes were isolated as described previously (17) or by the modified procedure described below. In both procedures, folded chromosomes were purified by centrifugation on neutral sucrose gradients, and the existence of a nonintegrative plasmid complex was inferred by subsequent detection of covalently closed circular (CCC) plasmid DNA (see below) from the folded chromosome fractions of the sucrose gradient.

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TABLE 1. *Bacterial strains*[a]

Bacterial strain	Genotype	Source or reference
CSH50	*ara(lac pro)rpsL thi*	Jeffrey Miller (23)
CSH51	CSH50(*ϕ*80d*lac*)	Jeffrey Miller (23)
CSH52	CSH51 *recA*	Jeffrey Miller (23)
BK209	CSH51 cured of *ϕ*80d*lac*	James Miller
BK210	CSH52 cured of *ϕ*80d*lac*, *recA*	James Miller
PCN24	CSH50 with mutation specifying elevated levels of certain plasmids	7
CR34	*thr leu thy* (2 μg/ml) *thi*	1
C600	*thr leu thi*	1
JC411	*arg pro leu met thy* (2 μg/ml) *rpsL*	D. Kingsbury
J5-3	*pro met rpsL*	D. Helinski
594	*sup gal rpsL*	N. Kleckner described in ref. 20
N3193	*lac gal rpsL*	H. Nijkamp

[a] Genetic symbols are described in references 2, 23, 25, 32, and 34.

TABLE 2. *Plasmids*[a]

Plasmids	Mol wt × 10^6	Genotype or comment	Source or reference
F	62	Classic sex factor of strain W1485	1
F'*lac*	95		
F'*lacI,P pro*$^+$*A,B*$^+$	145	Plasmid originally from strain CSH41 abbreviated in paper as F'*lac*$^-$ *pro*$^+$	23
F'Km	10.7	F replication genes and Km gene of R-factor	D. Helinski
pSC138(F'Ap)	10.4	F replication genes with *bla* gene from R-factor pI258	K. Timmis (36)
NR1	76	R-Tc, Cm, Sm, Su, *fi*$^+$	R. Rownd
R12	76	Plasmid copy number mutant of NR1	R. Rownd (24)
R64	76	R-Tc, Cm, Sm, Su, *fi*$^-$	D. Helsinki
P1*cml clr* 100	65	*ϕ*P1, thermoinducible, Cm	L. Rosner (29)
λN *sup* 7,53 CI857 *gal*$^+$	30	*ϕ*λ, maintained as plasmid; will not integrate into chromosome	N. Kleckner (31)
Clo DF 13	6	Cloacin production	H. Nijkamp
ColE1	4.2	Colicin E1 production	D. Helinski
pSC101	6	R-Tc	S. Cohen (6)
pSC134	10.2	Chimeric recombinant plasmid between pSC101 and ColE1	S. Cohen (36)
RSF1010	5.5	R-Sm, Su	S. Falkow
RSF1010-ColE1	9.7	Chimeric recombinant plasmid between RSF1010 and ColE1	B. Weisblum (33)

[a] Genetic symbols are described in references 2, 23, 25, 32, and 34. Resistances: Tc, tetracycline; Cm, chloramphenicol; Sm, streptomycin; Su, sulfonamide; Km, kanamycin; Ap, ampicillin.

Quantitative isolation of folded chromosomes is a difficult accomplishment when one uses the procedure originally described by Worcell and Burgi (37). Generally, this procedure results in liberation of membrane-associated folded chromosomes, which form large aggregates and pellet in the centrifugation step used to remove cellular debris (26). Additionally, the folded chromosome structures are quite unstable before the centrifugation step and frequently degrade themselves into chromosomal fragments. However, not all chromosomes are membrane associated nor are all associated chromosomes lost in centrifugation. With luck, 70% of the chromosomes in a lysate can be recovered in the supernatant after centrifugation; however, a more usual figure in our experience is 30 to 40% recovery (unpublished data).

A simple resolution of these difficulties came in an unexpected way. Our original discovery that plasmid-folded chromosome complexes exist was questioned on the basis that it might be an artifact of the lytic procedure. Marvin Stodolsky pointed out to us that, since the Worcel-Burgi procedure maintains a low ionic strength and uses ethylenediaminetetraacetate during spheroplast formation, theoretically the "nuclear" volume should expand. If in the expanded state the chromosome overlaps any resident plasmid DNA, then with the subsequent shift to high ionic strength medium during exposure to detergent the chromosome volume is expected to

shrink and might thereby entrap any overlapped plasmid DNA. To prevent this, Stodolsky suggested constant exposure of the cells to 1 M NaCl at all stages of the lytic procedure. We found subsequently that the fraction of a plasmid population complexed to its host chromosomes is constant regardless of when cells are exposed to high ionic strength medium during the lytic procedure (see Table 5). However, just as importantly, we also found that lysates made with 1 M NaCl present throughout the procedure do not degrade their folded chromosome structure when incubated at 25°C for up to 30 min; furthermore, such lysates routinely contain 70 to 95% of their folded chromosomes disassociated from membrane (see Fig. 1) since the chromosomes sediment with average *S* values of about 1,600 to 1,800 (8, 27). This means such lysates can be layered directly onto sucrose gradients without the intervention of a low-speed centrifugation step to remove cellular debris.

Ryder and Smith (30) described a different lytic procedure that effectively duplicates the desirable results of the high-salt modification. However, we find that their procedure is not applicable to a wide variety of strains.

In some cases, the high-salt modification fails. Other modifications of the Worcel-Burgi or Ryder-Smith procedures that should be tried in these cases are (i) adjustment of all solutions to pH 9.0, (ii) addition of digitoxin (0.8%, wt/vol) to the Brij-deoxycholate detergent solution, (iii) substitution of Sarkosyl or Triton X-100 for Brij 58, or (iv) various combinations of i, ii, and iii and the high-salt modification. Also, bacteria grown in minimal medium frequently give higher yields of membrane-released folded chromosomes. Which particular modification is best must be determined empirically case by case. In general, however, we find that the high-salt modification alone is the method of choice.

It is a curious observation that the amount of membrane-released folded chromosomes obtained by a given lysis procedure is a function of the plasmid species harbored by that host. For example, our CR34(F^+) or F^- strains yield up to 70% membrane-released folded chromosomes (17), whereas strain CR34(P1Cm) at best yields only 20% membrane-released folded chromosomes. Other examples too numerous to cite also exist. We speculate that the high-salt modification allows good release of folded chromosomes in lysates of CR34(P1Cm) probably because a long period of exposure to detergent at 25°C is possible without loss of the folded chromosome structure.

Detection of CCC plasmid DNA. The CCC form of plasmid DNA present in various regions of preparative sucrose gradients of the type shown in Fig. 1 or in crude lysates was determined either by the alkaline sucrose gradient (17) or by the dye-CsCl centrifugation technique (28). Invariably, we observed that the dye-CsCl procedure gave higher recoveries of CCC DNA compared with recoveries from alkaline sucrose gradients.

Calculation of the fraction of a plasmid population complexed to folded chromosomes. Method A. In our first approach to this determination, we measured the ratio of the percent CCC DNA in 1,800S folded chromosomes (equivalent to pool III of Fig. 1) to the percent CCC DNA in crude lysates. If the ratio is unity, the entire plasmid population can be considered bound to folded chromosomes; if it is less than unity, this value indicates the fraction of plasmids bound.

The results in Tables 3 and 7 showed that this approach is appropriate when the majority of the plasmid population is bound or when the majority of the plasmid population is unbound.

Method B. A more complete method of determining plasmid distribution in a sucrose gradient (and therefore association with folded chromosomes) is to analyze the entire gradient for CCC plasmid DNA. To do this, we pooled defined regions of a sucrose gradient into five groups, as indicated by the bars on Fig. 1. Each of the five pools was then examined for the amount of CCC DNA by the dye-CsCl procedure. To calculate the bound plasmid, it is only necessary to calculate the fraction of the plasmid population that is "freely" sedimenting; the difference from unity represents the bound fraction. The "freely" sedimenting plasmid population occurs in the pool V region of the gradient (0 to 300S sedimentation range). However, some of this plasmid is there spuriously due to breakage of folded chromosomes during the extraction and handling procedures. To correct for this, we measured the amount of chromosomal DNA in pools IV and V (the sedimentation range over which degraded chromosomes are found), multiplied this value by the percentage of CCC plasmid DNA normally associated with chromosomal DNA as found in pool III, and subtracted this amount of CCC DNA from the total CCC DNA found in pools IV and V. This corrected value was then divided by the total amount of CCC DNA found in all five pools to give the fraction of free plasmid DNA. In some analyses, the sucrose gradients were subdivided into only three regions and pooled. In the nomenclature of Fig. 1, these regions are equivalent to: (A) I plus II, (B) III, and (C) IV plus V. All of the plasmids in region C of these gradients, minus those that arose from broken chromosomes, are taken to be the nonassociated or freely sedimenting plasmids.

RESULTS

Since chromosomes sediment at rates that are about 10 to 90 times faster than the rates for free CCC plasmid DNA, plasmid-chromosome association can be inferred by showing that CCC plasmid DNA cosediments with its host's folded chromosomes in neutral sucrose gradients (Fig. 1 and Table 3). Thus, cosedimentation represents a relatively fast and convenient assay for association between replicons. The assay can also be used to quantitate the fraction of a CCC plasmid population non-integratively associated to chromosome (see method A, above).

In our initial results (Table 3), we found that all plasmid systems examined associate non-integratively. Furthermore, plasmid systems

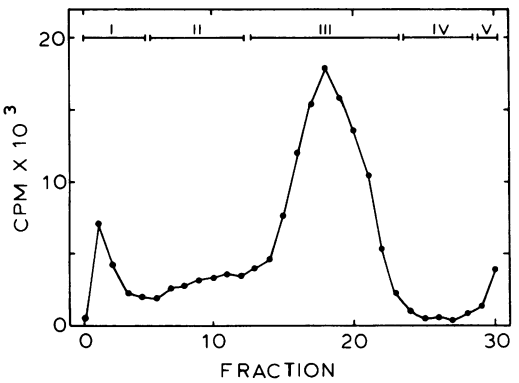

FIG. 1. *Whole-gradient distribution of plasmid DNA—a typical example. Bacterial strain JC411(E1) was lysed by the high-salt modification of our lytic procedure to release folded chromosomes from spheroplasts. The lysate was incubated at 25°C for 25 min, and then 0.3 ml was layered onto a 4.5-ml 10 to 30% neutral sucrose gradient (17) formed over a 0.5-ml cushion composed of a 70% sucrose solution. The gradient had been formed and maintained at 4°C. The layered lysate was then centrifuged in an SW50.1 rotor for 25 min at 17,000 rpm at 4°C. After centrifugation, 180-μl fractions were collected in tubes, and 10-μl aliquots were spotted on chromatography paper, washed, and counted (17) to determine the distribution of ^{3}H-labeled DNA in the gradient. Subsequently, the five regions of the gradient indicated by the Roman numerals were pooled separately, and the absolute amount of CCC DNA in each pool was determined by ethidium bromide-CsCl centrifugation. In almost all cases, the recovery of DNA was ≥90% of the material originally applied to either the sucrose or the CsCl gradient. In those instances where recoveries were less than 90%, the tests were repeated until high recoveries were obtained. However, in no instance did poor recovery result in an erroneous conclusion about plasmid distribution. Thus, DNA losses always were random.*

that are maintained at low numbers of plasmids per chromosome (F, F' NR1, and R64) are more highly associated than plasmids maintained at high numbers per chromosome (Clo DF13 and R6K). Also, the *recA* gene product does not seem to participate in association because an F'*lac* plasmid complexes identically in isogenic *recA*$^+$ and *recA*$^-$ hosts.

The data presented in Table 3, however, show ambiguities. For example, we frequently observed a higher percentage of CCC DNA in folded chromosome than in crude lysates. Moreover, we observed very little difference between the amounts of CCC DNA in crude lysates and folded chromosomes but consistently found an enrichment for freely sedimenting plasmids NR1 (only in strain CSH50) and R64 in the 0 to 300S range of the gradient. This enrichment should not occur unless a fraction of the plasmid population is not associated with folded chromosomes. These findings indicated to us that the method A for quantitating the degree of plasmid association has a low but perhaps significant uncertainty.

To reduce this uncertainty, we developed a modified method of analysis (method B) in which the entire sucrose gradient is assayed for plasmid DNA. Representative results are shown in Table 4. The uncertainty in whole-gradient analysis on repeated tests of large plasmids, such as R6K and F, was found to be

TABLE 3. *Association of plasmids with 1,800S nucleoids*

Host and plasmid	% CCC DNA[a] in:			% Associated[b]
	Crude	1,100–2,200S	0–300S	
CR34(F)	1.0	1.0	1.2	>90
BK209(F'*lac*)	1.6	1.8	2.5	>90
BK210(F'*lac*)	1.6	1.7	2.4	>90
CSH50(F'*lac*$^-$ *pro*$^+$)	1.5	1.3	1.6	>90
CSH50(PlCm)	1.4	1.5	1.6	>90
CSH50(NR1)	1.4	1.7	16.0	High
CR34(NR1)	1.3	1.8	2.3	>90
J5-3 (R64)	1.4	1.5	8.1	High
594(λN *sup* 7,53 CI857 *gal*$^+$)	3.2	1.8	11.0	57
CR34(R6K)	8.7	5.1	15.0	60
CSH50(R6K)	5.7	9.2	16.0	High
N3193(Clo DF13)	2.6	0.5	31.0	20
JC411(ColE1)	2.0	0.13		6.5

[a] Values are averages of at least two determinations in each case. Deviations are usually less than ± 0.2%, except in the 0 to 300S fraction in the samples that show enrichment for CCC DNA, where the deviations can be much greater because of the variation in the amount of linear DNA pooled.

[b] Association is calculated by method A as described in the text. Percent DNA is calculated as described in Table 4.

about 10% (Table 5). Unfortunately, this level of precision does not allow us to ascertain whether or not the enhanced levels of CCC DNA in pools IV and V for CSH50(NR1) (Table 5) and R64 (Table 3) are significant. We point out, however, that these indications of enrichment for freely sedimenting plasmid are qualitatively reproducible and much greater than those observed for other stringent plasmids such as F, F'*lac*$^-$ *pro*$^+$, and P1Cm as shown in Table 5.

In performing whole-gradient analysis on plasmids whose molecular weights are quite low, it is very difficult to detect CCC plasmid DNA in a particular region of the gradient in which both the absolute amount of DNA and the plasmid/chromosome ratios are low (for example, pool II, Fig. 1). Nonetheless, it is still possible to show gross differences in plasmid distributions for different plasmid systems. When we analyzed plasmids in the molecular weight range of 4×10^6 to 12×10^6 (Table 6), we found that the stringently controlled plasmids F'Km and F'Ap are associated with folded chromosomes to the same degree (90%) as the larger, stringently controlled plasmids (Tables 3 and 5). In contrast, plasmids under relaxed control and/or present in multicopy pools gener-

TABLE 4. *Plasmid distribution determined by whole-gradient analysis*[a]

Host and plasmid	Determination	Sample[b]						Corrected % associated
		Crude	I	II	III	IV	V	
N3193(Clo DF13)	A[c]	5,205	769	2,369	3,613	2,656	16,731	
	B[d]	186,539	133,975	342,173	538,019	20,500	66,171	
	C[e]	2.6	0.56	0.69	0.67	12.9	25.3	
	D[f]		3.0	9.0	13.8	10.2	64	26
CR34(R6K)	A		45,206	10,100	34,700	2,970	40,000	
	B		557,966	110,151	452,300	20,820	85,803	
	C		8.1	9.1	7.9	14.2	46	
	D		34	7.6	26.0	2.2	30	74

[a] Whole-gradient analysis refers to method B analysis given in the text. Bacteria were lysed by our original procedure (17).

[b] Sample refers to origin of radioactivity, i.e., crude lysate or fraction of crude lysate processed as described in the legend of Fig. 1. The Roman numerals refer to the region of the sucrose gradient pooled as indicated in Fig. 1. Each pool was then mixed with buffer, dye, and CsCl and sedimented to equilibrium to separate CCC plasmid DNA from other DNA forms.

[c] A, Counts per minute in plasmid peak of dye-CsCl gradient.

[d] B, Counts per minute in chromosome peak of same dye-CsCl gradient.

[e] C, Ratio of A/B expressed as a percentage.

[f] D, Plasmid counts of the pool expressed as a percentage of total plasmid counts found in pools I through V, i.e., the plasmid distribution.

TABLE 5. *Distribution of plasmids determined by whole-gradient analysis*[a]

Host and plasmid	Lysis[b]	% CCC DNA[c] in:			Corrected % associated
		Crude	Folded chromosome	Pools IV and V	
CSH50(F)	MS		2.1, 2.5	2.1, 1.5	91, 94
CSH50(F)	WB*	1.6	1.8	4.0	86
CSH50(R6K)	WB	4.7	10	43	82
CSH50(R6K)	MS	5.7	9.2	23	92
CR34(R6K)	WB	10	7.9	43	75
PCN24(F'*lac*$^-$ *pro*$^+$)	MS	5.4	5.5	5.4	100
CSH50(P1Cm)	MS	1.7	1.9	2.1[d]	100
PCN24(P1Cm)	MS	4.2	5.8	5.9[d]	100
CR34(NR1)	WB*	1.6	2.2	13.5	77
CR34(R12)	WB*	2.6	5.5	21	85

[a] Distributions are calculated by method B as described in the text.

[b] All bacterial strains were grown in M9 medium plus Casamino Acids. The letters marked with an asterisk indicate strains grown in M9 medium in which Casamino Acids was replaced by the required amino acids. WB, Lysis by our original procedure (17). MS, Lysis by the high-salt modification described in the text.

[c] Percent CCC DNA calculated as described in Table 4.

[d] Percent CCC DNA calculated for pool V only.

TABLE 6. *Distribution of small plasmids determined by whole-gradient analysis*[a]

Strain and plasmid	Mol wt × 10^6	Plasmid distribution (%)		Plasmids per 1,800S chromosome
		Associated	Free	
CR34(F'Km)[b]	10.5	86	14	1.5
CSH50(F'Ap)	9.4	93	7	1.6
CR34(pSC134)	10.2	26	74	1.5
C600(RSF1010-ColE1)	9.7	20	80	1.7
C600(pSC101)	6.0	48	52	1.3
C600(RSF1010)	5.5	27	73	1.8
JC411(ColE1)	4.2	8	92	0.4
N3193(CloDF 13)	6.0	26	74	2.8

[a] See Table 4 for examples of whole-gradient analysis.
[b] All lysates were made by using the high-salt modification, except for the F'Km analysis. In this case, our original procedure (17) was used.

ally associate poorly (20 to 30% of their populations) with folded chromosomes; pSC101 is perhaps an exception.

It is quite tempting to conclude from certain data presented in Table 6 that for some relaxed or multicopy plasmids (for example, pSC101 versus pSC134) there is correlation between plasmid size and the extent to which a plasmid population associates with folded chromosome. We caution against doing this because our data on distribution for these smaller plasmids simply are not that precise. We do, however, find it somewhat striking that all of the small plasmids listed in Table 6 seem to bind about two plasmid copies per 1,800S chromosome as determined from the percentages of plasmid CCC DNA found in folded chromosomes (data not shown). ColE1 seems to be an exception to this rule, but we also point out that one or two ColE1 molecules per 1,800S chromosome are quite difficult to detect in dye-CsCl gradients and, therefore, the error is large. ColE1 probably also associates about two molecules per chromosome (see Table 7). In conclusion, two salient points are firm from the data of Table 6: (i) a fraction of all small plasmids does cosediment with folded chromosomes; (ii) the fraction of a stringently controlled plasmid population that cosediments is high, whereas the fraction for a relaxed or multicopy plasmid population is low, irrespective of plasmid sizes.

Next we asked whether or not the fraction of a plasmid population of a given genotype associated with folded chromosomes varies as the number of plasmids per chromosomal equivalent varies. Plasmid R12 is a mutant form of plasmid NR1 (24) that is elevated at least twofold in the CSH50 host. This change in plasmid copy number does not substantially alter plasmid complexing to chromosomes (Table 5). A similar situation has been found for the wild-type PlCm and F'*lac*$^-$ *pro*$^+$ plasmids (Table 5), which are elevated three- and fivefold, respectively, in a mutant of CSH50 (7).

Very dramatic increases in plasmid copy numbers per chromosomal equivalent can be achieved by inhibiting protein synthesis in ColE1 strains (4). In two experiments, we exposed a logarithmic-phase culture of JC411(E1) to 180 μg of chloramphenicol per ml or starved it for required amino acids. Next, the amount of CCC DNA in purified folded chromosomes or in crude lysates was determined at various times after inhibition of protein synthesis. The results (Table 7) show that ColE1 plasmids do accumulate in membrane-released folded chromosomes generated in amino acid-starved bacteria and in membrane-bound folded chromosomes generated in chloramphenicol-treated bacteria. Using method A for analysis, we calculated that approximately 24% of the ColE1 plasmid population is bound to folded chromosomes over a wide range of plasmid/chromosome ratios, irrespective of the method used to amplify the ColE1 plasmid populations. The simplest interpretation of this finding is that the ColE1 plasmids are in equilibrium between the associated and the free state. It is not possible to determine from our experiments the physicochemical basis for this equilibrium.

So far, we note that modulation upward of plasmid copy numbers for R12, F'*lac*$^-$*pro*$^+$, PlCm, and ColE1 does not significantly alter the percentage of a plasmid population that associates with its host's folded chromosomes. This suggests an interesting idea: that the degree of association may be an inviolate feature of a given plasmid's properties. If this is true, it may indicate a fundamental role for plasmid association to folded chromosomes in the stable maintenance of plasmids. On the other hand, a constant percentage of bound plasmids is easily rationalized as manifesting nonspecific entrapment in chromosomes.

DISCUSSION

The principal conclusion from the data of Tables 1-6 is that plasmid complexing to folded chromosomes is a universal condition of plasmid existence in *E. coli* strains. Furthermore, we have found that the cryptic plasmid (molecular weight, 60 × 10^6) population found in *Salmonella typhimurium* LT-2 and which is under stringent control is likewise completely complexed with its host's folded chromosomes (22a). We may even tentatively extend nonintegrative complexes to eukaryotic systems since Green and Brooks (11) found that simian virus 40 CCC DNA forms cosediment at 1,000 × *g*

TABLE 7. *Cosedimentation of ColE1 plasmids with folded chromosomes purified from cells during inhibition of protein synthesis*[a]

Hours of inhibition	Treatment	% CCC DNA[b]		% CCC DNA associated with FC	Plasmids per FC
		Total	In FC		
0		0.5	0.138	27.6	0.8
2	−AA	2.16	0.42	19.4	2.5
4		3.64	0.94	25.8	5.6
6		5.64	1.26	22.3	7.5
0	+CM	1.3	ND		
3		11.8	3.1	26.2	18.1
				Mean = 24.3 ± 1.5 SE SE = SD/$\sqrt{5}$	

[a] AA, Casamino Acids; CM, chloramphenicol; ND, not detectable; SE, standard error; SD, standard deviation.

[b] Fractions of JC411(E1) cells were taken from cultures after the indicated physiological manipulation. Folded chromosome lysates were made at 25°C by the high-salt procedure, and then lysates were centrifuged on 10 to 30% neutral sucrose gradients. The folded chromosomes from the bacteria shifted to M9 medium lacking Casamino Acids sedimented at an average rate of about 1,600S; those from the culture containing 180 μg of CM/ml sedimented in a broad peak at rates of 4,000 to 6,000S. Pooled, folded chromosomes from each sucrose gradient and a fraction of each crude lysate were then analyzed for their respective amount of ColE1 CCC DNA by the dye-CsCl procedure. The percent ColE1 CCC DNA is expressed as in Table 4. The percent ColE1 DNA associated with folded chromosomes was calculated by method A.

with the chromatin of their host cells. It remains to be shown, however, if prokaryotic and eukaryotic CCC DNA-chromosome complexes are more than superficially similar.

In our efforts to show complexing in different plasmid systems, we opted to try for reasonably accurate estimates on the degree of complexing within each system. To do this, we chose the dye-CsCl gradient technique as an assay for CCC plasmid DNA because it is a sensitive method well suited for survey-type work. Furthermore, most workers assume that the CCC plasmid form represents the majority plasmid species in vivo for the systems we examined. Unfortunately, a number of circumstances thwart preservation of the CCC DNA form during handling; hence, analysis by the dye-CsCl technique underestimates the true amount of plasmid DNA (7). Basically, however, we feel that such losses of CCC DNA are likely to be systematic. Thus, even though CCC DNA levels might fluctuate, the actual distributions should not. To a reasonable approximation this is seen to be the case for both F and R6K as reported in Table 5. As shown in this table, the uncertainty in distribution values for these two plasmid systems spreads over a range of 15 to 20%. This uncertainty makes analysis of the intermediate distributions shown in Table 6 tenuous. Hence, our data are most valid for discerning systems that are either largely complexed or noncomplexed and should be viewed as such.

We caution that, in the very strictest sense, our usage of the verb "complex" to rationalize the phenomena of CCC plasmid-folded chromosome cosedimentation is not totally justified because complexing implies a specificity of association not unequivocally proven here. Our data in this paper, however, do firmly show cosedimentation of folded chromosomes with disparately sized plasmid replicons. That cosedimentation might be an artifact is still a possibility.

However, several lines of evidence argue against artifactual association of plasmids to folded chromosomes. First, we have already shown that CCC F DNA does not associate with folded chromosomes if added to spheroplasts before or after lysis by detergent (17). In similar types of reconstruction experiments, we have added the F'*lac pro*$^+$ and P1Cm plasmid separately to spheroplast solutions before and after lysis by detergent and never observed the subsequent cosedimentation of these plasmids with folded chromosomes (7; unpublished data). Second, plasmid entrapment in folded chromosomes does not seem to occur because of abrupt changes in chromosome structures resulting from the changes in cell permeability and ionic strength, which are features of certain lytic procedures (see Materials and Methods and Table 5).

Third, as can be seen in Table 4, membrane-associated and membrane-free folded chromosomes (identified by different sedimentation rates [27]) have the same percentages of CCC plasmid DNA. This is not only true for the two

plasmids shown in Table 4 but for all of the other plasmid systems examined in this study (data not shown). The simplest interpretation of these findings is that membrane does not randomly mediate plasmid association to folded chromosomes, since, if this were true, the membrane-bound chromosomes would have a greater number of associated plasmids compared with their membrane-free counterparts.

Fourth, in our efforts to characterize the molecular basis of association, one of us (J.R.M.) showed that F plasmid-chromosome association is stable during sedimentation through sucrose gradients containing either 2 or 200 μg of ethidium bromide per ml (data not shown). Thus, at the dye-DNA equivalence point (2 μg/ml [37]) where the supercoiled turns are zero, nucleoids and plasmids are converted to open circular structures and the nucleoid volume is greatly expanded. At this point, one would expect entanglement to be minimized and significant amounts of entrapped plasmid DNA to be separated from chromosome during centrifugation; as mentioned above, this does not happen. Likewise, the R6K plasmid-chromosome association is also stable during sedimentation through gradients containing either 2 or 20 μg of ethidium bromide per ml (M. Wlodarczyk, unpublished data).

Fifth, we see from our data that the degree of plasmid association appears to depend very much on the plasmid type and not on the plasmid size. Quite the opposite might be expected if entrapment were significant because a larger plasmid molecule would have a greater chance of entanglement in a nucleoid. This point is typified by comparing the relative association of plasmids F'Ap, F'Km, pSC134, and RSF 1010-ColE1 to folded chromosomes (Table 6). These results are inconsistent with plasmid association being strictly a function of plasmid molecular weight.

Sixth, we note that plasmids such as NR1, R64, R6K, Clo DF13, and ColE1, which show significant amounts of nonassociated members (especially in pool V), also segregate well into minicells (13, 14, 19, 21, 22), whereas F, F', and P1 plasmids, which show little free CCC DNA, segregate poorly into minicells (15). This correlation is also consistent with our general conclusions about the qualitative distribution of these plasmids between their bound and unbound states within their host cells.

A final argument for specific association is the fact that all stringently controlled plasmids associate completely with folded chromosomes, whereas relaxed and/or multicopied plasmids associate poorly. In exception to this general rule, we observe that the relaxed plasmid R6K (18) associates the majority of its population to host chromosomes. The significance of this exception is not readily apparent but may reflect an inviolate feature of an ancestral form of R6K that may have been under stringent control (therefore highly associated) and is still retained by the present-day R6K plasmids.

In contrast to these arguments against artifacts, the fact that the absolute numbers of F *lac*$^-$*pro*$^+$, P1Cm, and ColE1 plasmids that bind to folded chromosomes increases (Tables 5 and 7) when plasmid copy numbers are modulated upward by genetic or physiological changes argues either for plasmids actively binding at multiple chromosomal sites or for nonspecific entrapment. Data to resolve these possibilities are lacking.

All in all, we conclude that nonintegrative plasmid-chromosome complexes are a general phenomenon. There is yet no definitive proof to answer the question of whether or not they represent artifacts. An answer to this question is desirable, however, because if plasmid-chromosome complexes exist in vivo, it is easy to imagine (17) how they may significantly influence the processes of plasmid replication, segregation, genetic recombination, and cointegrate formation, as well as responses to curing agents.

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