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Retention of Episomes During Protoplasting and During Propagation in the L State

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ABSTRACT

KAWAKAMI, MASAYA (Georgetown University, Washington, D.C.), AND OTTO E. LANDMAN. Retention of episomes during protoplasting and during propagation in the L state. J. Bacteriol. **92**:398–404. 1966.—In earlier work, it was observed that the mesosomes of *Bacillus megaterium* and *B. subtilis* are expelled from the cell interior during protoplasting and that mesosome fragments are released into the supernatant fluid as the cell wall disintegrates. Since the resultant protoplasts remain intact and capable of reproduction, the expelled contents of the mesosome "bag" are presumably external to the protoplast membrane and nonessential to survival. Accordingly, if episomes (plasmids) were localized in the extramembrane mesosome "bag," it would be predicted that protoplasting would cure cells of their episomes. This prediction was tested in three different systems: *B. subtilis* W23 carrying phage SP-10, *B. megaterium* 216 carrying megacinogenic factors A and C, and *B. megaterium* C4M$^-$ carrying megacinogenic factor C. No curing due to protoplasting was observed. Even during propagation in the L form, when septation is not functioning and the distribution of deoxyribonucleic acid to daughter cells is severely disrupted, curing was not observed in any of the above systems nor in *Salmonella* L forms carrying F^0 *lac*. It is concluded that episomes are located at a position on the interior side of the cell membrane and that their distribution to daughter cells is coordinated with that of the chromosome.

A variety of considerations have led to the idea that bacterial episomes in their extrachromosomal state might be associated with the cell membrane or with localized portions of the cell membrane. Important among these considerations are the emerging analogies between episomal replicons and the chromosomal replicon, coupled with suggestive recent indications that the chromosomal replicon may indeed be attached to the membrane. Evidence for such an attachment comes from Ganesan and Lederberg's observation (6) that, in *Bacillus subtilis*, most of the nascent deoxyribonucleic acid (DNA), defined by a 2 to 10 sec H^3-thymidine pulse, is separated from the bulk DNA during fractionation and pellets with a membrane fraction. Analogous findings for *Escherichia coli* were reported earlier by Hanawalt and Ray (7) and others. Further, electronmicroscopy of the expulsion of mesosomes from *B. subtilis* during hypertonic treatment or protoplasting has shown that the bacterial nucleoid is pulled to the cell periphery along with the mesosome—presumably by the DNA-membrane attachment site (13, 14).

Even before the preceding observations had been reported, Jacob, Brenner, and Cuzin postulated that, in F$^+$, Hfr, and R factor-containing strains, the episomes were located at a membrane site at the equatorial perimeter of the cell (9). This postulate was made partly to account for the efficient transfer of replicons during conjugation; this efficiency could be understood more readily if one assumed that the replicons were situated at a location near the point of origin of the mating tube. Further, it was hypothesized, peripheral attachment sites for the bacterial replicons would serve to regulate the distribution of chromosomes and episomes to the daughter cells after replication. Since bacteria lack a mitotic mechanism, the attachment points would serve both as centromeres and centrioles.

Electron microscopy of protoplast formation in gram-positive bacteria (5, 14) suggested a further experimental approach to the question of episome localization. It was observed that an

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important fraction of the membrane system of the bacteria—the mesosome—was ejected from the cells and lost into the supernatant fluid during cell-wall removal. When the protoplasts were subsequently grown in the wall-less state as L colonies, the mesosomes did not re-form (14). If the episomes were attached to the surface of the mesosome which faces the cell exterior, or if they were localized within the mesosome bag, it would be predicted that the episomes would be damaged or ejected along with the mesosomes during protoplasting.

In a preceding publication, our initial experiments in this research area were reported (10). It was shown that mild penicillin treatment of a *Salmonella paratyphi* B strain carrying the resistance transfer factor episome R_{11} resulted in a high incidence of loss of episome markers. [According to revised bacteriological nomenclature, *S. paratyphi* B should be designated as *S. schottmuelleri* (3).] The "curing" effect of penicillin was not observed in *E. coli* carrying the same episome. All observations were made on the spheroplast-like reverting L forms which retain the outer cell wall layers (16; Landman, Burchard, and Angelety, Bacteriol. Proc., p. 58, 1962). In this spheroplast system, any curing effect of the cell wall-attacking reagent, penicillin, cannot clearly be attributed to the expulsion of mesosomes or mesosome-like structures, since the intact outer wall layers would presumably prevent the escape of any mesosome fragments and allow rapid repair of the cells and their mesosomes after withdrawal of penicillin or of lysozyme.

In the present paper, the ambiguity of the spheroplast state is avoided by studying the fate of episomes in cells which have been stripped entirely of their cell walls. The combinations of bacterial host strains and extrachromosomal elements used are shown in Table 1.

In contrast to the spheroplasts examined for the presence of R by Kawakami and Landman (10), the protoplasts and L bodies listed in Table 1 are believed to be devoid of cell wall. Further, the gram-positive protoplasts and L forms of systems 1 and 2 are presumed to have lost their mesosomes in the course of cell-wall removal (5, 14). In the case of system 3, the gram-negative *S. schottmuelleri*, the conformation of internal membranes in the bacillary and L states is not known.

The classification of the extrachromosomal elements of Table 1 as "episomes" is arbitrary, since there is no positive evidence that any of them interact directly with the bacterial chromosome. The designation "plasmid" would be equally appropriate (1). However, it is clear from earlier work and from the experiments to be reported that these elements can be present or absent from the cell, and that they generally behave as non-chromosome-associated replicons.

TABLE 1. *Experimental systems studied*

System	Host bacterium	States of host strain examined for presence of extrachromosomal element	Extrachromosomal element
1	*Bacillus subtilis* W23S^r	Bacilli Protoplasts L forms	Phage SP-10 (2), a "carried" phage
2a	*B. megaterium* 216	Bacilli Protoplasts L forms	Megacinogenic factors A and C (8)
2b	*B. megaterium* C4M$^-$	Bacilli L forms	Megacinogenic factor C (8)
3	*Salmonella schottmuelleri*	Bacilli L forms	F^0 *lac*

STRAINS AND MEDIA

System 1. B. subtilis W23S^r, a streptomycin-resistant derivative of strain W23, was used as indicator for phage SP-10 (15). A carrier strain, carrying phage SP-10 in loose association, was derived from W23S^r by picking from a turbid SP-10 plaque.

Spores were grown on Oxoid nutrient agar (Consolidated Laboratories, Chicago Heights, Ill.) containing 5 g/liter of NaCl. After 2 days at 37 C and 3 days at room temperature, sporulation was nearly complete. The spores were suspended in water, heated at 60 C for 30 min, and further freed of debris by repeated centrifugation at 1,500 × *g*. Vegetative cells were grown on Difco Blood Agar Base and in Difco Nutrient Broth. The liquid SL2 medium used for protoplasting was described previously, as was the solid medium SD, used for plating of protoplasts (12). The gelatin medium for the induction of rapid quantitative reversion of protoplasts to the bacillary state had the following composition: 0.5 M sodium succinate (*p*H 7.6); K_2HPO_4, 0.7 g/liter; KH_2PO_4, 0.3 g/liter; glucose, 2 g/liter; NH_4NO_3, 1 g/liter; $CaCl_2 \cdot 2H_2O$, 0.15 g/liter; $MgCl \cdot 6H_2O$, 1.17 g/liter; $MnCl_2 \cdot 4H_2O$, 0.05 g/liter; Difco gelatin, 250 g/liter. PA medium, used for the assay of phage SP-10, was described by Thorne (15). Antiphage serum was a gift of Martha Taylor-Stedman. Its neutralization velocity *K* value was 3,698.

System 2. B. megaterium strain 216, producing megacins A and C, strain C4M$^-$, producing megacin C, and strain MUT/SR, used for the assay of both megacins (8), were kindly furnished by I. B. Holland. When a streptomycin-resistant, megacin-sensitive,

strain was needed for our experiments on megacinogen infection, a new streptomycin-resistant derivative of strain MUT/SR was isolated by the method of Holland and Roberts (8), since the strain in our collection had lost its streptomycin resistance.

NBY medium had the following composition: Difco Nutrient Broth, 23 g/liter; Difco Yeast Extract, 2 g/liter; agar, 15 g/liter. SL1 medium and SL2 medium were described previously (12). DP medium, which was used for the plating of *B. megaterium* protoplasts, had the following composition: 0.5 M sodium succinate (*p*H 7.0); 0.005 M $MgCl_2$; K_2HPO_4, 3.5 g/liter; KH_2PO_4, 1.5 g/liter; glucose, 2 g/liter; acid-hydrolyzed casein (Nutritional Biochemicals Corp., Cleveland, Ohio), 1 g/liter; agar, 8 g/liter; L-tryptophan, 0.02 g/liter; horse serum, 5 ml/liter. ONA medium was Oxoid Nutrient Agar (8).

System 3. The *S. schottmuelleri* used here was identical to *S. paratyphi* B 777 used earlier in our laboratory (11).

Medium KL has the following composition: agar, 9 g/liter; Difco Nutrient Broth, 8 g/liter; Difco Yeast Extract, 2 g/liter; glucose, 1 g/liter; 0.003 M $CaCl_2$; NaCl, 16.7 g/liter; horse serum, 50 ml/liter; horse blood cell lysate, 0.74 g/liter. The lysate was produced by adding 10 volumes of sterile distilled water to packed horse blood cells; 25 ml of this lysate was used as a supplement per liter of KL medium.

RESULTS

Retention of phage SP-10 by B. subtilis W23S^r upon protoplasting and during propagation in the L state. The initial aim of our experiments was to determine whether the protoplasting process itself led to the ejection of the carried phage. To test for the possible curing effect of protoplasting, as distinguished from curing due to growth in the L state, the protoplasts were plated on gelatin reversion medium immediately after wall removal. On this medium, reversion occurs without prior multiplication in the protoplast (L) state. The bacillary colonies emerging on gelatin medium were then checked for phage content as soon as possible.

Maintenance of the carrier state of phage SP-10 in *B. subtilis* strain W23S^r is normally contingent upon the reinfection of spontaneously cured cells by free phage (2, 15). Since protoplasts lack cell wall and hence phage attachment sites, reinfection cannot occur in these forms. To provide comparable conditions for the bacillary controls, antiserum was included in all experiments to neutralize supernatant phage as soon as it was produced.

For the experiment shown in Table 2, the following procedure was used. Spores of a carrier culture were inoculated onto a Blood Agar Base plate and incubated at 37 C overnight. Vegetative cells from this plate were suspended to a concentration of 10^8 per milliliter in 5 ml of Nutrient Broth containing a 1:500 dilution of antiserum and shaken vigorously for 20 min at 37 C to remove all phage; 1 ml of this suspension was pipetted into 9 ml of the sucrose-containing SL2 medium to which a 1:500 dilution of antiserum and 500 μg/ml of lysozyme had been added. After 30 min of static incubation in a water bath (32 C), more than 99% of the cells had been converted to protoplasts. The protoplast suspension was then plated on SD medium, and on gelatin reversion medium. SD medium permits a quantitative assessment of the effectiveness of the protoplasting, since protoplasts form L colonies on this medium after 2 days of incubation at 30 C, while rod forms produce bacillary colonies during this time. On gelatin reversion medium, the protoplasts rapidly revert to the bacillary form upon incubation at 26 C (12). When the revertant colonies on the gelatin plates had grown to about 1 mm in diameter, they were counted, and individual clones were picked with sterile tooth picks and inoculated onto PA plates to be checked for their phage content. In each case, about one-half of the cells in these protoplast-derived clones still contained phage. The transferred colonies on PA medium were allowed to grow for 48 hr. They were then covered with a few drops of a suspension of phage-sensitive W23S^r bacteria in soft agar and incubated further at 37 C (overnight). Plaques in the localized soft-agar layer indicated that the protoplast-derived clone still contained phage. Control data were obtained with cells which had been handled in the same way except for the lysozyme treatment.

The experiment shown in Table 2 and others like it clearly showed that protoplasting did not eliminate carried phage from their host bacteria. Moreover, protoplasting even failed to enhance the very considerable spontaneous phage-elimination rate.

Having obtained this result, we now posed a second, somewhat related question: Would the rate of episome elimination be greater during propagation in the L state, when distribution of protoplasm and nuclei among viable units is very uneven and erratic (14), and when the septum-forming mechanism—and hence, perhaps, the replicon-distribution mechanism—is inoperative? Thirty-five small agar blocks containing unreverted L colonies of W23S^r were excised from SD medium plates, placed "face down" on fresh SD medium, and dragged along the surface. Along the path of the "drag," secondary L colonies arose, representing the offspring of individual L bodies from the primary L colony. The secondary L colonies were allowed to revert, and 10 or more subcolonies from each primary colony were then examined with regard to their

TABLE 2. *Loss of SP-10 from Bacillus subtilis during protoplasting*

Incubation in SL2	Plating medium	Viable count		No. of colonies tested	Cells forming phage-negative colonies
		Bacillary colonies	L colonies		
					%
With lysozyme.....	SD	1.2×10^4	5.2×10^{6a}		
	Gelatin	2.3×10^{6a}		239	66.5[b]
Without lysozyme.....	SD	1.4×10^7	—		
	Gelatin	1.3×10^7	—	240	64.5[b]

[a] The lowered survival after lysozyme treatment is attributed to incomplete stabilization of W23S^r protoplasts.

[b] The spore suspension from which this cell population was derived contained 93% carrier cells.

carrier state on PA medium (Table 3). Parallel experiments were performed with control bacillary colonies. Clearly, the rate of curing is no higher during propagation in the L state than that during bacillary propagation on the same medium. The very high rate of curing, which becomes evident when this experimental method is used, is probably due to the fact that SP-10 reinfects W23S^r very inefficiently on SD medium, which contains 0.5 M sodium succinate (Kawakami and Landman, Bacteriol. Proc., p. 112, 1966). Growth on SD medium thus acts somewhat like growth in antiserum-containing medium for carrier strain W23S^r in being an effective means of curing (2).

Retention of megacinogenic factors by B. megaterium upon protoplasting and during propagation in the L state. Although protoplasting and propagation in the L state did not promote loss of SP-10 from *B. subtilis* W23S^r, it was considered important to carry out analogous experiments with other systems. Further experiments were especially appropriate in view of Bott and Strauss' evidence (2) which appeared to show that each *B. subtilis* cell contains many SP-10 replicons and that, consequently, curing-by-protoplasting might be unusually difficult in this system.

The strains chosen for our further study of curing-by-protoplasting were *B. megaterium* 216, producing megacins A and C, and *B. megaterium* C4M$^-$, producing megacin C (8). Attempts were made to define the episome status of these bacteriocinogenic factors more clearly by demonstrating infective transfer of megacinogenic factors A and C. In these experiments, a streptomycin-resistant, megacin-sensitive derivative of MUT/R was grown in mixed liquid culture with strain 216 or layered in an agar film over colonies of strain 216. Numerous strains which exhibited both megacin and streptomycin resistance were isolated and checked for the production of megacin. No evidence of megacinogen transfer was obtained. Attempts were also made to stimulate the rate of spontaneous megacinogen loss through growth in 0.02 to 25 μg/ml of acriflavine. In sampling a population of 392 cells, no increase in the rate of curing due to acriflavine was noted.

TABLE 3. *Elimination of SP-10 from Bacillus subtilis during propagation in the L state*

Type of colony	Total no. of primary colonies examined	No. of primary colonies showing at least one carrier subcolony	No. of primary colonies showing no carrier subcolony	Primary colonies testing as cured
				%
L.....	35	9	26	72
Bacillary..	40	11	29	73

Both strain 216 and strain C4M$^-$ can be protoplasted readily in the sucrose-containing stabilizing medium SL2, and both form colonies on DP medium which are very rapidly overgrown by revertants.

Strain 216 was grown overnight on NBY agar, suspended to a density of 3×10^7 colony-forming units/ml in SL1 medium, and incubated for 180 min at 37 C in a rotating test tube. The culture was then diluted 1:10 in SL2 medium and incubated an additional 90 min. Lysozyme was added to a concentration of 200 μg/ml, and incubation was continued in standing culture at 32 C. More than 99.9% of the cells had been converted to spheres 30 min after lysozyme addition. After 180 min of incubation in lysozyme, the cells were plated on DP medium. Upon overnight incubation at 30 C, the colonies were classified as bacillary, reverted L, or L, and reverted L colonies were transferred with a toothpick to NBY and ONA plates. The transferred colonies were incubated for 48 hr at 37 C; then each colony was assayed for production of megacin A (on NBY medium) and megacin C (on ONA

medium), by covering the irradiation-killed colonies with a soft-agar lawn of indicator strain MUT/SR (8).

No significant difference was observed between the rate of loss of megacinogenic factor "A" from protoplasted cells and from control cells (Table 4). None of the colonies from which megacin A production had been lost exhibited a megacin C halo; apparently, the A and C factors were always eliminated jointly.

Analogous experiments were carried out with the megacin C-producing strain C4M$^-$. No curing of the megacinogenic factor was observed among either the protoplasted or the control cells (Table 4).

Again, as in the case of the *B. subtilis* SP-10 system, an attempt was made to follow the elimination of megacinogenic factors as a consequence of propagation in the L state. Several L colonies of the A, C-producing strain which had not yet reverted (as judged by phase microscopy) were dragged on DP medium and incubated at 30 C overnight. The bacillary colonies developing from these L bodies (and appropriate control colonies derived from bacilli) were assayed for their capacity to produce megacin A. As in the case of the W23S^r–SP-10 system, it was found that episome curing was not significantly enhanced during propagation in the L state (Table 5).

Retention of the F° lac particle by S. schottmuelleri in the L state. Paralleling the work described above, experiments were carried out with F° *lac*-carrying *S. schottmuelleri* to determine whether episome curing would occur during conversion of these bacteria to the L form. In *S. schottmuelleri*, as in the other gram-negative species, lysozyme removes only a small portion of the cell wall. The spheroplasts which result from this enzyme treatment promptly revert to the bacillary form when lysozyme action is stopped. Apparently, a more far-reaching removal of cell wall is required to trigger establishment of the stable, heritably transmitted, L state (11; Landman et al., Bacteriol. Proc., p. 58, 1962). This thoroughgoing wall removal can be achieved in *S. schottmuelleri* by prolonged incubation at high penicillin concentration. The L colonies of *S. schottmuelleri* which are formed by 2 to 50% of the cells inoculated onto soft penicillin-serum-agar are quite stable; reversion to the bacillary form is a rare event and no effective means have been found so far to stimulate its occurrence (11).

In the *S. schottmuelleri*-F° *lac* system, there is thus no clearly defined protoplast state available for study. Instead, stable L forms were used to study episome curing. Further, because of the infrequency of reversion, tests for the presence of the F° *lac* factor were made on the L forms directly by assaying their content of β-galactosidase.

The F° *lac*-carrying strain of *S. schottmuelleri* used in these experiments is infectious for F° *lac*. However, attempts to cure it by overnight incubation in 0.8 to 12 μg/ml of acriflavine were unsuccessful.

The F° *lac* factor was introduced into *S. schottmuelleri* by infection from *S. typhosa*. An overnight culture of *S. schottmuelleri* (F° *lac*) on Nutrient Agar was suspended in saline and plated on medium KL containing 200 units/ml of penicillin G. After 10 days of incubation at 30 C in petri dishes sealed with dish seals (Fisher Scientific Co.), the survivors (about 1 in 14 of the inoculated bacteria) had given rise to stable L colonies. Individual colonies were picked up and placed on membrane filters (HA type; Millipore Filter Corp., Bedford, Mass.). The filters were then left at 4 C for 30 min on plates containing 2% agar, 1.67% NaCl, and 0.05 M

TABLE 4. *Elimination of megacinogenic factors A and C from lysozyme-treated and control cells of Bacillus megaterium*

Strain	Static incubation at 32 C	Viable count[a]		No. of colonies	Cured colonies
		L-colony formers	B-colony formers		
					%
A + C producer (216)..	None	—	4.1×10^6	142	11.3
	Without lysozyme (180 min)	—	4.6×10^6	106	12.2
	With lysozyme (180 min)	6.4×10^5	1.6×10^6	147[b]	12.9
C producer (C4M$^-$)....	None	—	3.1×10^6	150	0
	Without lysozyme (180 min)	—	4.2×10^6	101	0
	With lysozyme (180 min)	5.8×10^5	1.2×10^6	107[b]	0

[a] Due to the instability of the L state of *B. megaterium* on DP medium, 85 to 95% of the "L colonies" already contained both L bodies and bacilli when they reached macroscopically visible size.

[b] Among the lysozyme-treated cells, only reverted L colonies were tested.

TABLE 5. *Elimination of megacinogenic factor "A" during propagation in the L state*

State of cells during propagation	Primary colony no.	No. of secondary colonies examined	No. of cured colonies among secondary colonies	Secondary colonies cured[a]
				%
Bacillary	1	52	4	10.7
	2	11	0	
	3	24	10	
	4	43	3	
	5	49	5	
	6	64	4	
L	1	13	1	17.2
	2	18	6	
	3	34	7	
	4	9	0	
	5	21	3	
	6	21	3	

[a] $\chi^2 = 0.237$; $P = >0.1$. In an earlier, smaller-scale experiment, the percentage of cured colonies was higher among the bacillus-derived colonies.

phosphate buffer (*p*H 6.8). This procedure permits diffusion of contaminating colored medium constituents. The membranes were then impregnated with a solution of 660 μg/ml of *o*-nitrophenyl galactoside in 0.05 M phosphate buffer (*p*H 6.8) and incubated at room temperature in a petri dish together with moist filter paper. A yellow color developed within 30 min at sites inoculated with β-galactosidase-positive colonies, owing to the liberation of free *o*-nitrophenol. The F° *lac* element permits β-galactosidase to form constitutively. Of 618 L colonies tested, none had lost the F° *lac* element. L colonies of *S. schottmuelleri* devoid of F° *lac* served as controls.

To trace the fate of the F° *lac* element during propagation in the L state, 12 individual L colonies were dispersed on penicillin-free KL agar and their subcolonies were allowed to grow for 14 days at 30 C; 23 to 25 subcolonies of each of the 12 were then assayed for β-galactosidase. All of the 296 secondary L colonies were β-galactosidase-positive.

Although neither conversion to the stable L form nor propagation in the L state stimulated the loss of the F° *lac* episome, it was still possible that the overall episome content of L forms differed from that of the parent bacillary strains. Quantitative assay of the β-galactosidase content of bacilli and their homologous stable L forms did not reveal a consistent pattern. In four tests involving two strains of *S. schottmuelleri*, the specific activity of the β-galactosidase of the L forms ranged from 41 to 93% of that of the homologous bacillary form.

DISCUSSION

In the experimental systems employed here, removal of the cell wall either by lysozyme treatment or by protracted incubation at high penicillin concentrations did not result in episome loss, or in the stimulation of the spontaneous rate of episome loss. Clearly, our initial question "are episomes localized in the expelled portion of the mesosomes?" can be answered in the negative for these systems. The behavior of the episomal replicons thus parallels the presumed behavior of the chromosomal replicon. Since the bacterial population remains fully viable during protoplasting, we already know that pieces of the chromosome are apparently neither torn off nor expelled during protoplasting. The present experiments indicate that the episomal replicons are similarly preserved.

In our view, these results do not constitute evidence against a DNA-mesosome attachment hypothesis. They do indicate, however, that if such attachment sites exist, they are located at the surface of the mesosome which faces the cell interior. Further, these sites would have to be quite rugged to withstand the trauma of mesosome expulsion; perhaps the portion of the mesosome "bag" which harbors them is not expelled during protoplasting but is stretched out to become part of the peripheral protoplast membrane.

It should be underlined here that even the assumption that episomes are attached to membranes rests on an insecure foundation. Alternative hypotheses—for example, the possibility that episomes are linked directly to the chromosome—cannot be ruled out at present.

Even after we had failed to demonstrate stimulation of curing by protoplasting, we anticipated that we would find curing during L-form growth. This expectation was based principally on electron microscopic observations which had shown that the presumed DNA distribution mechanism —the mesosomes-cum-septa—was completely absent in the L form (14). Further, the actual DNA distribution among the L bodies was quite irregular. In our view, the apparently chaotic fragmentation of cytoplasm during L growth into large, small, DNA-containing, and DNA-free bodies should surely produce nucleated cells without episomes, unless a special associative mechanism existed which kept the chromosomal and episomal replicons together. We believe that our failure to find curing provides evidence for such an associative mechanism. Strong support

for the same conclusion was recently presented by Cuzin and Jacob (4), who showed that episome and chromosome replicas labeled in a given replication cycle remain together in the same cell for many subsequent cell generations. The observations reported in this paper indicate that this associative mechanism persists even in the L state, that is, even in the absence of mesosomes and under circumstances when the mechanism which normally distributes DNA to cells is severely disturbed. We agree with Cuzin and Jacob that this associative mechanism may well be an attachment to a common membrane.

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