Genet. Res., Camb. (1976), **27**, *pp.* 161–170
With 1 *plate and* 3 *text-figures*
Printed in Great Britain

Covalently closed, circular DNA in kappa endosymbionts of *Paramecium*

By JUDITH A. DILTS*

Department of Zoology, Indiana University, Bloomington, Indiana 47401, U.S.A.

(*Received* 10 *October* 1975)

SUMMARY

Caedobacter taeniospiralis (kappa), a bacterial endosymbiont isolated from *Paramecium tetraurelia* stock 51, contains, in addition to the bacterial chromosome, covalently closed circular DNA molecules as shown by isolation on dye-buoyant-density gradients. The closed circular molecule has a contour length of 13·75 ± 0·04 μm with a buoyant density of 1·698 g/cm³. The buoyant density of the bacterial chromosome is 1·700–1·701 g/cm³. Kappa of the 51 group isolated from stock 298 and stock 6g2, *P. tetraurelia*, also contain the closed circular DNA. Two forms of kappa coexist in paramecia: brights and nonbrights. Examination by density-gradient centrifugation of the DNA of brights and nonbrights shows the extrachromosomal DNA to be associated mainly with brights. It is suggested that the extrachromosomal DNA might be the determinant for the refractile bodies and the helical phage-like structures found in brights.

1. INTRODUCTION

Caedobacter taeniospiralis (kappa), a bacterial endosymbiont found in certain stocks of *Paramecium biaurelia* and *P. tetraurelia*, is responsible for the killing phenotype first described by Sonneborn (1938). Kappa has two forms in paramecia: nonbrights and brights. Nonbrights are the reproductive and infective forms of kappa and can, when induced, give rise to brights. Brights, the particles responsible for the killing, contain refractile bodies (R bodies) which are coiled ribbons of protein, and, on the inside end of the coiled R body, either spherical or helical phage-like structures. Spherical phage-like structures isolated from the kappa of stock 562, *P. biaurelia*, were found to be composed of about equal amounts of protein and DNA, supporting the view that they are bacteriophages (Preer, Preer, Rudman & Jurand, 1971). The helical phage-like structures, like those in stock 51, *P. tetraurelia*, have not been isolated to date but are thought to be abortive subunits of the spherical phage-like structures (Preer, Preer & Jurand, 1974).

The spherical phage-like structures are generally present whenever R bodies are present as demonstrated by electron microscopy of the kappa from stocks 7 and 562, *P. biaurelia* (Preer & Jurand, 1968; Grimes & Preer, 1971). The actual relationship was clarified when it was found that the R body and capsid proteins of stock

* Present address: Department of Biology, William Jewell College, Liberty, Missouri, U.S.A.

562 kappa were antigenically related, suggesting a common genetic determinant (Singler, 1974). The same relationship is assumed between R bodies and the helical phage-like structures. Experiments also showed that the stock 562 R bodies cross-reacted antigenically with R bodies from stock 51 (Singler-Bastiaans, 1975). This finding points out the probable relationship between R bodies of different kappas and lends support to the idea that the helical phage-like structures are simply incomplete components of the spherical phage-like structures.

Preer & Preer (1967) have hypothesized that nonbrights harbour a prophage or plasmid which when induced directs the synthesis of the R body and phage-like structures. Support for this theory was shown by induction with ultraviolet light of nonbrights to brights in stock 51 kappa (Preer, Rudman, Preer & Jurand, 1974) paralleling ultraviolet induction of the prophage of lysogenic phage and of certain bacteriocins.

As plasmids and prophages of some defective phages exist as covalently closed, circular molecules (Helinski & Clewell, 1971), it was thought possible that such molecules might exist in kappa. C. Kung and Y. Suyama in a preliminary examination with the electron microscope of stock 51 kappa DNA found a few open circular DNA molecules of about 20 μm in length (personal communication to J. R. Preer, Jr.).

The following paper confirms by dye-buoyant-density centrifugation the existence of covalently closed, circular DNA in stock 51, stock 298 and stock 6g2 kappa and describes electron microscope and density centrifugation observations on the DNAs from 51 kappa. Evidence is also presented concerning the role of the extrachromosomal DNA as the determinant for the R body and defective phage. A preliminary report of this work has been published (Dilts, 1974).

2. MATERIAL AND METHODS

(i) *Strains used*

Kappa-containing strains of *Paramecium tetraurelia* used in the experiments which follow are listed below with a brief description of their origin and characteristics:

stock 51, standard strain: kappa-containing strain originally isolated from a stream in Spencer, Indiana; T. M. Sonneborn collection.

71M7-61: a derived *P. tetraurelia* multiple marker stock; wild-type kappa introduced by a cross with stock 51; T. N. Sonneborn collection.

d4-88: a derived *P. tetraurelia* multiple marker stock; wild-type kappa introduced by a cross with stock 51; T. M. Sonneborn collection.

high bright: stock 51 originally containing wild-type kappa; kappas maintain a high proportion of brights, about 32% (Preer *et al.* 1974); J. R. Preer, Jr. collection.

low bright: stock 51 originally containing wild-type kappa; kappas maintain a low proportion of brights, about 5% (Preer *et al.* 1974); J. R. Preer, Jr. collection.

stock 298: isolated from Empire Range, Panama; contains 51-type kappa (Preer *et al.* 1974); T. M. Sonneborn collection.

stock 6g2: isolated from Twin Lakes, Bloomington, Indiana; contains 51-type kappa as determined by killing phenotype; W. G. Landis collection.

(ii) *Culture methods*

Paramecia were grown initially either on *Klebsiella pneumoniae* (*Aerobacter aerogenes*) in Cerophyl (Sonneborn, 1970) or in bacteria-free medium containing *Chlamydomonas reinhardi* (Preer, Rudman, Preer & Jurand, 1974). Those grown in bacterized Cerophyl were fed for two fissions per day at 27 °C until 4 days before harvesting. In order to increase the number of kappa per host cell and the proportion of brights to nonbrights, the cultures were then fed for one fission per day for 3 days and finally allowed to starve for 24 h prior to harvesting. The final volume of the cultures was 25–35 l.

Initial growth in bacteria-free medium was employed in an attempt to reduce the number of contaminating bacteria in the cultures. The paramecia, grown initially on *C. reinhardi*, were sterilely transferred in 1 ml aliquots to sixteen 3000 ml flasks containing 1500 ml of bacterized Cerophyl medium and were then incubated at 27 °C. When the bacteria were exhausted from the medium, 500 ml of bacterized Cerophyl were added to each flask. The paramecia were harvested 2 days later.

Packed paramecia, 4–8 ml, containing approximately 10^7 animals per ml, were harvested and homogenized, and the kappa were isolated by means of anion exchange epichlorhydrin triethanolamine cellulose (ECTEOLA) columns as described by Sonneborn (1970).

(iii) *Counts of nonbrights and brights*

To determine the numbers of nonbrights and brights a known amount of the kappa suspension was added to a known amount of standardized yeast cells (2×10^8/ml). Yeast cells, nonbrights and brights were counted using bright phase microscopy until 20 yeast cells had been recorded. Five such counts were generally recorded for each sample and 95% confidence limits determined.

(iv) *Separation of brights and nonbrights by sucrose gradient centrifugation*

When necessary for subsequent comparison of their DNAs, separation of brights and nonbrights was accomplished by sucrose gradient centrifugation. Isolated kappa were centrifuged at 48 200 *g* for 5 min and resuspended in 0·5 ml of 0·01 M sodium-potassium phosphate buffer at pH 7 (pH 7 buffer). The kappa suspension was then layered on a continuous 9 ml, 5–40% sucrose gradient and centrifuged at room temperature in an International centrifuge, Model HN, at about 3300 rev/min for 20 min. The gradient was generally divided into four fractions. Equal volumes of pH 7 buffer were added to the fractions and they were centrifuged at 48 200 *g* for 5 min. After resuspending each in 1 ml of pH 7 buffer and removing a small sample for subsequent counts of brights and nonbrights, the fractions were centrifuged as

before and resuspended in 0·5 ml saline-EDTA (0·15 M-NaCl, 0·1 M EDTA, pH 8·0). DNA isolation was then carried out by the phenol-ribonuclease method described below.

(v) *Reagents*

Sarkosyl, NL-97, K & K Laboratories, Inc.; caesium chloride, optical grade, Penn Rare Metals Division of Kawecki Berylco Industries, Inc.; ethidium bromide, Sigma Chemical Company.

(vi) *Preparation of DNA*

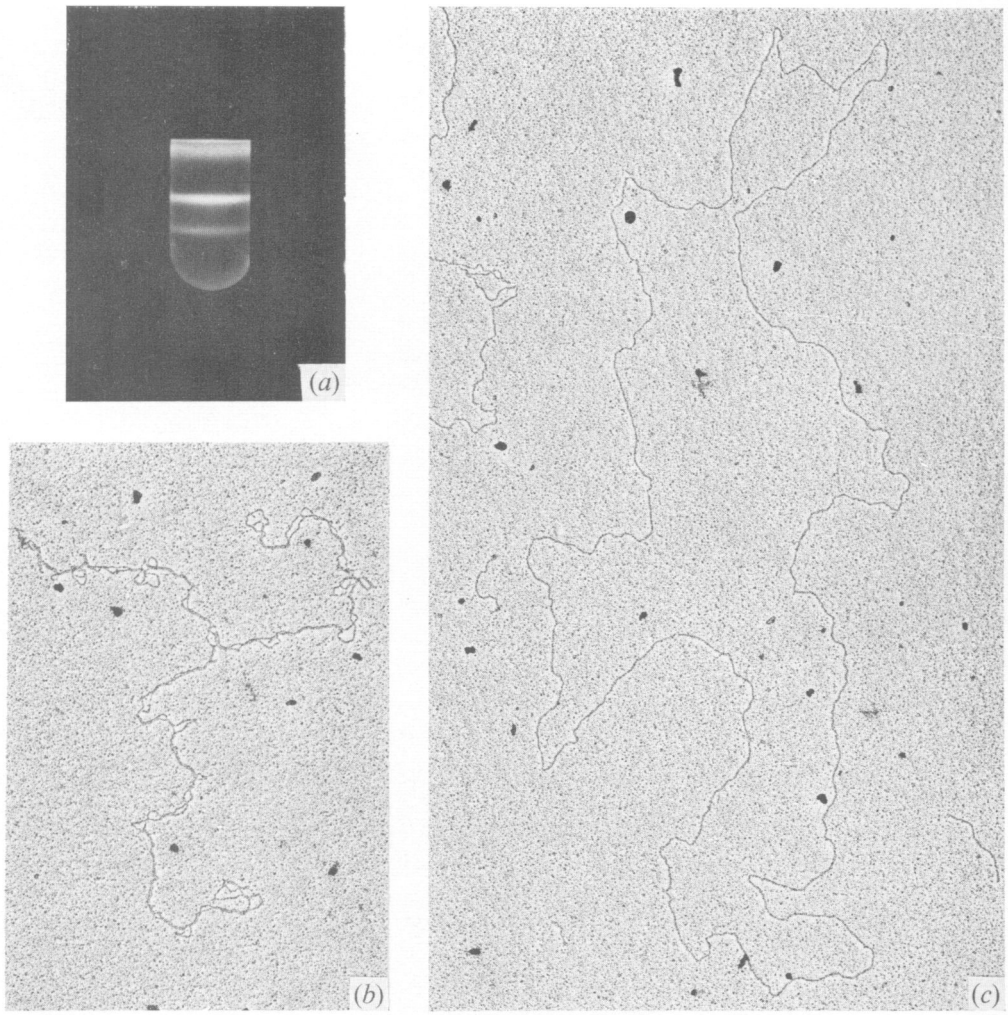
The DNA was isolated from 10^8–10^9 kappa by one of two methods. The first involved isolation and purification with phenol and ribonuclease. Isolated kappa in pH 7 buffer were centrifuged at 48200 ***g*** for 5 min and resuspended in 1 ml of saline-EDTA. An equal volume of either 2% sodium lauryl sulphate (1 part 10% SLS: 4 parts saline-EDTA) or 1% sarkosyl (1 part 10% sarkosyl: 9 parts saline-EDTA) was added to the suspension and the mixture allowed to sit at room temperature for 10 min. Isolation and purification of the DNA was carried out according to Suyama & Preer (1965). After DNA isolation and purification the optical density at 260 nm was determined in a Beckman DB-G grating spectrophotometer and the DNA stored at 4 °C.

The second method employed was the preparation of sarkosyl lysates by a procedure similar to that described by Bazaral & Helinski (1968). Isolated kappas were centrifuged at 48200 ***g*** for 5 min. The pellet was suspended in 1·7–1·8 ml of saline-EDTA, and an equal volume of 1% sarkosyl was added. The mixture was allowed to stand at room temperature for 10 min and was then centrifuged for 3 min at 48200 ***g***. The supernatant was recovered and brought up to 3·8 ml with saline-EDTA. The lysate was then readied for dye-buoyant-density centrifugation.

(vii) *Dye-buoyant-density centrifugation*

Dye-buoyant-density centrifugation was performed according to the method of Radloff, Bauer & Vinograd (1967) with modifications by J. Sinclair (personal communication). The purified DNA ($OD_{260} = 0{\cdot}5 - 10{\cdot}0$), in a volume of 3·8 ml of BPES (0·006 M-Na_2HPO4, 0·002 M-NaH_2PO4, 0·001 M-Na_2 EDTA and 0·179 M-NaCl) or a similar volume of sarkosyl lysate, was put into a polyallomer tube ($\frac{5}{8} \times 3$ in.) with 3·4 ***g*** of CsCl and 0·08 ml of 1% ethidium bromide in water. The contents were overlaid with paraffin oil to fill the tube. Samples were centrifuged at 45000 rev/min in a Beckman Model L preparative ultracentrifuge for 39–44 h at 44 °F in a Type 65 Fixed-Angle Rotor.

After centrifugation, the ultracentrifuge tubes were examined in a darkened room with a long-wave Blak-Ray black light lamp UVL-21. Black and white photographs were taken with Kodak Recording Film 2475 through a combination of an Ultraviolet Products contrast (Model J-344) filter and a Kodak Wratten No. 16 gelatin filter (Watson, Bauer & Vinograd, 1971). Ten-drop fractions were collected using a needle pierced through the bottom of the tube. For removal of the ethidium bromide, the separate fractions were dialysed against 2000 ml of BPES at 4 °C with two 2000 ml changes.



(*a*) Results of dye-buoyant-density centrifugation of stock 51 kappa DNA. Two DNA bands are apparent in the centrifuge tube. The top major band is linear DNA. The second, denser band is covalently closed, circular DNA. (*b*, *c*) Electron micrographs of (*b*) a supercoiled DNA molecule and (*c*) an open circular DNA molecule isolated by dye-buoyant-density centrifugation from the DNA of kappa of strain HB. × 34400.

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(viii) *Electron microscopy*

The DNA was prepared for electron microscopy by the microversion spontaneous adsorption method of Lang & Mitani (1970). Staining with uranyl acetate in methanol and subsequent removal of collodion from the grids was performed according to Gordon & Kleinschmidt (1968). The grids were rotary shadowed with 80:20 platinum:palladium (Ladd Research Industries, Inc.) at an angle of 8° and a distance of 3 in. from the source of the metal (personal communication from J. Gall). Specimens were examined in a Philips EM 300 electron microscope, and micrographs were taken at about 9550×. A grating replica of a 54864 Line Block was used for calibrations. Micrographs of open circular DNA molecules were enlarged to a final magnification of 168000–173000× with a Simmon Omega enlarger, projected on paper and traced. The contour length of the enlarged molecules was then measured with a map measurer and the actual contour length determined.

(ix) *Density gradient centrifugation*

The buoyant density of the kappa and/or plasmid DNA was determined by centrifugation in a CsCl density gradient, using a Beckman Model E analytical ultracentrifuge, at 44770 rev/min for 20 h at 25 °C. *Micrococcus lysodeikticus* DNA (supplied by J. Sinclair) was used as the reference (density taken as 1·731 g/cm^3). Samples contained 3 μg of reference DNA, 2–3 μg of unknown DNA, BPES to bring the sample to 0·8 ml and 1 g of CsCl. Ultraviolet absorption photographs were taken of most of the runs, and the negatives were scanned on a Beckman Analytrol densitometer. Determinations on other runs were done using the photoelectric scanning system of the ultracentrifuge.

3. RESULTS

(i) *Stock 51 kappa*

(*a*) *Isolation of covalently closed, circular DNA*. The DNA of stock 51 kappa was subjected to ethidium bromide–caesium chloride centrifugation which separates covalently closed, circular DNA from linear or nicked circular DNA (Radloff *et al.*, 1967). Two DNA bands were apparent in the gradient after centrifugation (Plate 1*a*). The major band was shown by electron microscopy to consist mainly of long, linear DNA strands of variable length. In view of the length of the strands and the fact that the band represents a major fraction of the total kappa DNA, this band is presumed to consist primarily of kappa chromosomal DNA. The second, denser band contained covalently closed, circular DNA strands (see below). The two bands were found both in purified DNA and in the sarkosyl lysate of stock 51 kappa.

Stock 51 kappa also appears in a number of strains which have been derived from stock 51 and kept separated for a number of years. In assessing the significance of the circular DNA it seemed desirable to see whether it was a constant feature of these strains. Accordingly an examination was made of 71M7-61, d4-88, HB (high bright) and LB (low bright) strains. Both linear and circular bands were found in all these strains except LB. Strain LB, of course, had a low percentage of brights

(1–4 %), compared to the usually much higher percentages (up to near 50 %) in different cultures of the other strains. This finding suggested that the circular DNA is derived primarily from bright kappas and that samples of LB contained too few of them for visualization of the circular DNA. In fact, counts of the numbers of brights in the LB cultures tested were near the lower limit for the numbers needed to give a circular band with the other strains (about 5×10^7).

(*b*) *Configuration and contour length of the extrachromosomal DNA*. Examination of the covalently closed, circular DNA band showed both supercoiled molecules and the open circular molecules which result when a nick occurs in one of the DNA strands during storage, thereby allowing the supercoiled circles to unwind (Plate 1*b*, *c*).

The class of open circular molecules observed was homogeneous in length. Measurements of 18 open circular molecules from strain HB disclosed a mean contour length for the plasmid DNA of 13·75 μm with a standard error of 0·04 μm. Using a value of $2{\cdot}07 \times 10^6$ daltons per μm (Lang, 1970) the contour length corresponds to an approximate molecular weight of $2{\cdot}8 \times 10^7$ daltons.

(*c*) *Buoyant densities of the linear and closed circular DNAs*. The results of buoyant density determinations on DNA isolated from preparations of 51 kappa containing both brights and nonbrights show a main band of DNA at 1·700–1·701 g/cm^3 and a satellite band at about 1·698 g/cm^3. The satellite band, depending upon the preparation, can be observed as either a skew in the main peak, a shoulder or a separate peak. Fig. 1(*a*) is a densitometer tracing of DNA isolated from a kappa preparation of strain HB. The reference DNA, *Micrococcus lysodeikticus*, is the peak of highest buoyant density (1·731 g/cm^3). The main kappa DNA peak is at 1·700 g/cm^3 while the satellite band can be seen, in this case, as a distinct peak at 1·698 g/cm^3.

The identity of the two DNA bands was elucidated by buoyant density analyses of the 51 kappa closed circular, plasmid DNA and the linear chromosomal DNA which had been previously separated by dye-buoyant-density centrifugation. The buoyant density of the circular DNA was determined to be 1·698 g/cm^3, accounting for the satellite band. The chromosomal DNA was found to have a buoyant density of 1·700–1·701 g/cm^3 corresponding to the main DNA peak observed in the buoyant density analyses of the whole kappa DNA. These densities held for all strains examined.

The buoyant density analyses of the dye-isolated chromosomal DNA also showed that it was contaminated with a small amount of plasmid DNA. The contaminant is believed to be nicked circular or linear plasmid DNA which separates in ethidium bromide with linear DNA.

Densitometer tracings of the chromosomal and plasmid DNAs of strain HB kappa are shown in Fig. 1(*b*) and (*c*). In both tracings the reference DNA, *M. lysodeikticus*, at 1·731 g/cm^3, is the peak of higher buoyant density. The chromosomal DNA (Fig. 1*b*) with a density of 1·700 g/cm^3 has a slight skew in the peak towards a lower density indicating the presence of a small amount of plasmid DNA. The plasmid DNA (Fig. 1*c*) is seen as a uniform peak at 1·698 g/cm^3.

The difference in buoyant density between the plasmid and chromosomal

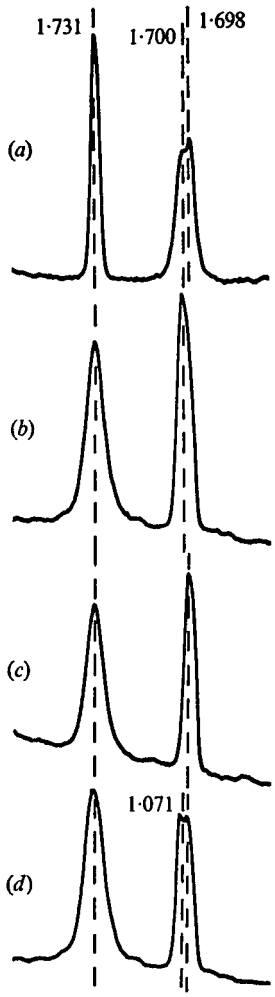

Fig. 1

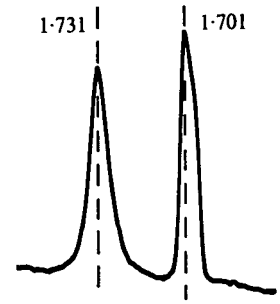

Fig. 2

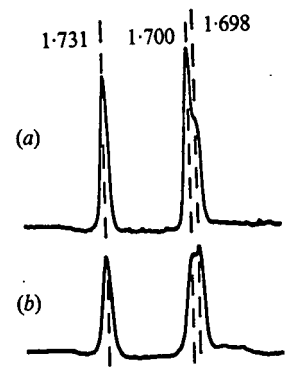

Fig. 3

Fig. 1. Densitometer tracings from buoyant density determinations of HB kappa DNA. The band at the left is the reference DNA, *Micrococcus lysodeikticus*, at 1·731 g/cm³. (*a*) DNA from a kappa preparation of brights and nonbrights. The main kappa peak is 1·700 g/cm³ while the satellite band is a distinct peak at 1·698 g/cm³. (*b*) Linear, chromosomal DNA originally isolated by dye-buoyant-density centrifugation. The DNA bands at 1·700 g/cm³. (*c*) Closed circular plasmid DNA originally isolated by dye-buoyant-density centrifugation. The DNA bands at 1·698 g/cm³. (*d*) A mixture of isolated chromosomal and isolated plasmid DNAs. The chromosomal DNA bands at 1·701 g/cm³, the plasmid DNA at 1·698 g/cm³.

Fig. 2. Densitometer tracing from a buoyant density determination on the chromosomal DNA from the kappa of strain LB. The DNA bands at 1·701 g/cm³. The reference DNA, *M. lysodeikticus*, is the band on the left at 1·731 g/cm³.

Fig. 3. Densitometer tracings from buoyant density determinations of DNA isolated from (*a*) a preparation of HB kappa containing 9·7 % brights and (*b*) a preparation of HB kappa containing 56·5 % brights. The band at 1·700 g/cm³ is kappa chromosomal DNA while the band at 1·698 g/cm³ is plasmid DNA. The reference DNA, *M. lysodeikticus*, is taken as 1·731 g/cm³.

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DNAs, although small, was reproducible. The difference is clearly seen in Fig. 1 (*d*) which shows the densitometer tracing of a gradient containing samples of both chromosomal and plasmid DNAs from the kappa of strain HB. Again, the chromosomal DNA bands at 1·701 g/cm^3, and the plasmid DNA bands at 1·698 g/cm^3.

As mentioned above, when the DNA of kappa of strain LB was examined by dye-buoyant-density centrifugation, only one band of DNA was detected. When, however, the DNA from the band was centrifuged in a caesium chloride gradient, the presence of a satellite band of DNA was detected by a skew in the main peak of DNA. The buoyant density of the main DNA peak was calculated to be that of the chromosomal DNA, 1·701 g/cm^3. The satellite band had a buoyant density of about 1·698 g/cm^3 and is, therefore, assumed to be the plasmid DNA. The densitometer tracing of the gradient is shown in Fig. 2.

(*d*) *Comparison of the DNA of brights with the DNA of nonbrights.* In order to determine whether the plasmid DNA was associated with brights or nonbrights, the DNAs of each were examined separately. Separation of bright and nonbright kappas from strain HB was achieved by sucrose gradient centrifugation. Four different sucrose gradient separations were performed. In each case the fraction containing a low percentage of brights and one containing a high percentage of brights were chosen for analysis. Table 1 shows the results of counts made for the two fractions in each of the four experiments. In each case (*a*) is the fraction containing the low percentage of brights and (*b*) the fraction containing the high percentage of brights. Total numbers of nonbrights and brights in each fraction and the calculated percentages of brights appear in the Table. In all cases the fraction with the lowest percentage of brights contained less than 10% brights while the fraction with the highest percentage contained greater than 40% brights.

When the DNA was extracted from the fractions and examined by density gradient centrifugation a difference could be seen in the proportion of chromosomal DNA to plasmid DNA. In every case with an increase in brights there occurred an increase in the amount of plasmid DNA in proportion to the amount of chromosomal DNA. The densitometer tracings shown in Fig. 3 of DNA samples from the high and low percentage fractions of experiment 2 clearly show the increase in plasmid DNA (1·698 g/cm^3) in the fraction with the higher percentage of brights (Fig. 3*b*).

(ii) *Stock 298 and stock 6g2*

A stock consists of the descendants of a single isolate from nature. To assess further the significance of the plasmid DNA it was desirable to examine other stocks containing 51-type kappa, i.e. kappa which cause pre-lethal aboral humps in sensitive paramecia, for the presence of the plasmid. Two kappa-containing stocks were examined, 298 and 6g2. Stock 298 was chosen because it had been isolated in Panama, a location which is far from the source of stock 51 (Spencer, Indiana). Stock 6g2 was chosen because the original isolation had recently been taken from nature (October, 1974) and it was useful to see if the plasmid still existed in nature in association with the presence of brights.

When the kappa from the stocks were examined by dye-buoyant-density centri-

fugation both linear and closed circular bands were observed as in stock 51 kappa preparations. Determinations of the buoyant densities of the two bands yielded densities of 1·700 g/cm³ for the linear and 1·698 g/cm³ for the closed circular DNAs. These densities are the same as those observed in stock 51 kappa.

Table 1. *Numbers of nonbrights and brights and percentage brights obtained by sucrose gradient separation in four experiments*

Experiment*	Nonbrights $\times 10^{-8}$†	Brights $\times 10^{-8}$†	Brights (%)
1 (*a*)	239	15	5·9
1 (*b*)	72	192	72·7
2 (*a*)	12	1·3	9·7 (7·3–12·4)
2 (*b*)	2·2	2·7	56·6 (46·4–66·4)
3 (*a*)	25	1·4	5·2 (2·8–8·3)
3 (*b*)	9·3	7·1	43·1 (36·6–49·7)
4 (*a*)	37	1·7	4·6 (0·43–12·8)
4 (*b*)	3·5	2·3	40 (34–45·5)

* In each experiment, (*a*) and (*b*) refer to the fraction containing the low or high percentage of brights, respectively. In Expt 1, as the actual number of kappa counted are presented, no confidence limits were determined. For Expts 2–4 the mean number of kappa in each fraction are given. 95% confidence limits are in parentheses for the % brights.

† Applies only to Expts 2–4.

4. DISCUSSION

Stock 51 kappa and other members of the 51 group have been shown to contain extrachromosomal DNA having a contour length of 13·75 ± 0·04 μm and a buoyant density of 1·698 g/cm³. It is unlikely that the DNA is simply a *Paramecium* mitochondrial or a bacterial contaminant. Mitochondrial DNA has been shown to be linear (Suyama & Miura, 1968) and to have a higher buoyant density (1·702 g/cm³; Suyama & Preer, 1965). The presence of enough contaminating bacteria to allow detection of a plasmid DNA should yield not only the plasmid, but also a bacterial DNA band in the caesium chloride gradient. *Aerobacter aerogenes*, present in every experiment, is the most likely source of a consistent plasmid band, but DNA with its buoyant density (1·715 g/cm³; Smith-Sonneborn, Green & Marmur, 1963) was never observed.

The role of the plasmid in 51-type kappa is as yet not completely verified; however, due to the association of the plasmid DNA with the bright kappa it is likely that the plasmid's function is not cryptic but rather that it is the genetic determinant for the R body and the helical phage-like structures. The discovery of the plasmid and its characteristics provide strong support for the hypothesis that nonbright kappa are lysogenic bacteria.

The author would like to thank Dr John R. Preer, Jr., for his guidance, encouragement and friendship during the course of this research and the preparation of the manuscript. This work is a portion of the dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Indiana University. Contribution 1007 from the Department of Zoology, Indiana University, Bloomington, Indiana. Supported by National Science Foundation grant GB-27609 and Public Health Service grant GM20038 to Dr John R. Preer, Jr., and by Public Health Service Training Grant 82 to the author.

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