The Size and Structure of the DNA Genome of Symbiont Xenosome Particles in the Ciliate *Paraurionema acutum*

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The size and structure of the DNA genome of xenosomes, bacterial endosymbionts of the marine hymenostome ciliate, *Paraurionema acutum* 110-3, were investigated. Renaturation kinetic measurements, determined optically and by hydroxyapatite chromatography, suggested a genome size of 0.34×10^9 daltons. Sedimentation rate measurements of DNA gently released from the symbionts yielded molecules of comparable size. The analytical complexity, determined chemically, was 3.03×10^9 daltons. Consistent with these and other data is a model for the structure of the symbiont genome in which the DNA exists in the form of nine circularly permuted, double-stranded DNA molecules of unique sequence, each of molecular weight 0.34×10^9 . It is suggested that xenosomes and certain symbionts found in ciliated protozoa may be extant forms of once free-living bacteria that have adapted to the intracellular environment.

INTRODUCTION

Self-reproducing bacterium-like symbionts, termed xenosomes, are found in the cytoplasm of the marine protozoan *Paraurionema acutum* (Soldi *et al.*, 1974). The symbionts, released from the host by gentle rupture, are capable of infecting homologous as well as heterologous strains of *Paraurionema* spp. and at least one other ciliate of marine origin, *Miamiensis avidus* Ma (Soldi & Brickson, 1978). The particles are also capable of killing other ciliate strains, especially those of the genus *Uronema* (Soldi & Brickson, 1978). Bacterium-like symbionts appear to be widespread in nature, particularly among the protozoa; it has been suggested that they may represent a new class of organisms that have adapted to an intracellular existence (Soldi, 1974). To further characterize these organisms with respect to their nature and possible phylogenetic origins we have investigated the structure of the DNA genome of xenosomes, derived from *P. acutum* 110-3, using renaturation rate kinetics, sedimentation rate data and electron microscopy.

METHODS

Culture methods. Xenosome-bearing *Paraurionema acutum* were cultured axenically in M medium at 27 °C in the dark. Mass cultures were prepared in 500 ml batches in 4-litre Fernbach flasks and harvested in the early stationary phase of growth (6 d). Under these conditions protozoan populations ranged from 0.8 to 1.2×10^6 ml⁻¹ and yields were 3–4 ml packed cells l⁻¹. The composition and preparation of M medium, maintenance of stock cultures, mass cultivation and harvesting procedures were described by Soldi & Merlin (1972). Xenosome-free protozoa were obtained by treating xenosome-bearing animals with tetracycline (100 µg ml⁻¹ for 7 d) to selectively destroy the symbionts.

Isolation and purification of xenosomes. Xenosomes were isolated and purified from xenosome-bearing protozoa by (1) a modification of a procedure previously described for the isolation of mu and pi symbionts of *Paramecium* (Soldi & Godoy, 1974), or (2) a procedure based on separation of the particles in discontinuous sucrose gradients. In the former method, instant sea water ($\rho = 1.015$ g cm⁻³) (ISW), (Soldi & Merlin, 1972) was used instead of migration salt solution (Soldi & Godoy, 1974). The latter method, which has not been described previously, was as follows: Xenosome-bearing *P. acutum* from 1–4 l of culture were collected in a de Laval continuous-flow centrifuge or by pelleting the animals at 1000 g for 5 min at 4 °C in 125 ml capacity conical

centrifuge bottles. After removal of the supernatant by aspiration, the pellets were suspended in 10 volumes of ISW and disrupted in a Waring blender. Completeness of cell breakage was verified by examination of samples of the homogenates in a phase microscope. The homogenate was then centrifuged at 1000 *g* for 5 min at 4 °C. The cloudy supernatant was transferred by pipette to 50 ml round-bottomed Nalgene tubes and centrifuged at 10000 *g* for 30 min at 4 °C. After discarding the supernatant by decantation, the pellets were suspended with the aid of a vortex mixer in three successive 2 ml portions of 0.5 M-sucrose prepared in ISW. The portions were combined in 12 ml capacity centrifuge tubes and centrifuged at 500 *g* for 2 min to remove aggregated material. The supernatant (approximately 6 ml) was then transferred to a $\frac{5}{8}$ " $\times$ 3" (16 $\times$ 76 mm) polyallomer tube. In successive portions, 2.5 ml each of 1.5 M- and 1.75 M-sucrose, and 1.0 ml 2.0 M-sucrose were sub-layered with the aid of a glass funnel whose stem was positioned so that its tip (pulled in a glass flame to approximately 2 mm in diameter) rested at a point 1–2 mm above the bottom of the tube; the sucrose solutions were prepared in ISW. The tubes were then centrifuged at 30000 r.p.m. for 1.5 h at 4 °C using a Spinco rotor SW-36 in a model L-65 B ultracentrifuge. Xenosomes deposited at the interface between the 1.75 M- and 2.0 M-sucrose layers, within the 2.0 M-sucrose layer and at the bottom of the tubes. Xenosomes at the interface and within the 2.0 M-sucrose layer were free of cellular and sub-cellular debris; those at the bottom of the tube contained refractile bodies. These bodies were shown previously to be excretory in nature and were composed mainly of guanine and hypoxanthine in insoluble form (Soldo *et al.*, 1978). Their presence did not interfere with subsequent extraction and purification of DNA from xenosomes. Yields of xenosomes from 1 litre of culture ranged from 1.5 to 2.0×10^{11} .

Extraction and purification of DNA. DNA was extracted from freshly isolated xenosomes by the procedure of Marmur (1961). DNA from freshly harvested xenosome-bearing and xenosome-free protozoa was extracted as described by Soldo & Godoy (1973). CsCl density gradient centrifugation was used for final purification of the DNA preparations. Yields of highly purified DNA from 2×10^{11} xenosomes ranged from 0.5 to 1.0 mg. Approximately 2–3 mg of highly purified whole-cell DNA was usually obtained from 1 litre of culture. We thank Dr Rudolf Werner of the University of Miami School of Medicine for the gift of bacteriophage T₄. The phage DNA was labelled with [³H]thymidine.

CsCl density gradient centrifugation. Analytical CsCl gradient centrifugation was carried out in a model E ultracentrifuge using samples containing 2–4 μ g DNA ml⁻¹. *Escherichia coli* K12 DNA was included in each run as a reference marker ($\rho = 1.710$ g cm⁻³). Preparative CsCl density gradient centrifugation was carried out using DNA samples containing 5–10 absorbancy units (260 nm) in 5.0 ml CsCl solution ($\rho = 1.700$ g cm⁻³) at 55000 r.p.m. for 19 h at 20 °C in a Spinco Vi 65 vertical rotor.

Thermal transition and renaturation. Thermal transition was followed by heating native DNA in standard saline citrate (SSC, containing 0.15 mol NaCl and 0.015 mol trisodium citrate l⁻¹) at a rate of 0.5 °C min⁻¹ during melting in a stoppered quartz cuvette (1 cm light path) in a Gilford model 250 spectrophotometer using the automatic thermo-programmer. Denaturation and renaturation of the DNA samples were accomplished as previously described (Soldo & Godoy, 1973). The reaction was followed at 260 nm.

Determination of renaturation rate constants and kinetic complexity. Rate constants were calculated from second-order renaturation rate plots (Wetmur & Davidson, 1968). Alkaline sedimentation coefficients were estimated by the method of Studier (1965) using alkaline sucrose gradients calibrated by the procedures described by Martin & Ames (1961). The kinetic complexity of the DNA samples was calculated from the renaturation rate constant and alkaline sedimentation coefficient using the formula of Wetmur & Davidson (1968), as previously described (Soldo & Godoy, 1973).

Kinetic complexity was also determined by hydroxyapatite chromatography (Britten & Kohne, 1965), essentially as described by Soldo & Godoy (1972) except that, during reassociation in 0.12 M-NaH₂PO₄ at 60 °C, samples were removed at shorter time intervals.

The genomic size of xenosome DNA was determined optically from second-order renaturation rate plots in SSC (Marmur, 1961) or 1 M-NaCl, and by hydroxyapatite chromatography in 0.12 M-NaH₂PO₄, pH 7.0 (Britten & Kohne, 1965). For these determinations the DNA was sheared by sonication to a single-stranded size of about 100000 daltons. Bacteriophage T₄ DNA, which has a G + C content of 34 mol%, almost identical to that of xenosome DNA, was included in each run as a reference standard and obviated the need to correct for G + C content (Wetmur & Davidson, 1968).

Sedimentation measurements of genome size. Sedimentation patterns of ¹⁴C-labelled xenosome lysates were determined in 30 ml 5–25% (w/v) linear sucrose gradients using the general procedures of Kavenhoff (1972). [³H]Thymidine-labelled bacteriophage T₄ was included in each run as a marker. For these experiments it was necessary to label symbiont DNA. Attempts to introduce labelled thymine and its derivatives into symbiont DNA yielded purified preparations of low specific activity, i.e., a few hundred c.p.m. (μ g DNA)⁻¹. [¹⁴C]Adenine, however, was an excellent precursor and it became possible to obtain symbiont DNA preparations with specific activities up to 35000 c.p.m. (μ g DNA)⁻¹. Xenosomes labelled with [¹⁴C]adenine were isolated and purified. They were gently lysed in the lysis mixture described by Kavenhoff (1972) (except that sodium lauryl sulphate was used instead of Sarkosyl to effect lysis) and subjected to sucrose gradient centrifugation. Using the equation of Burgi &

Hershey (1963): $(s_1/s_2) = (M_1/M_2)^{0.35}$, where s_1 and s_2 are the sedimentation coefficients of two molecules of molecular weights M_1 and M_2 , respectively. Values of s and M for T_4 are 57S and 0.13×10^9 , respectively (Leighton & Rubenstein, 1969).

Phase and electron microscopy. Aceto-orcein stained and unstained xenosome preparations were examined by phase microscopy as described previously (Soldo & Brickson, 1978). Contour lengths of DNA released from xenosomes were measured by the protein monolayer techniques of Kleinschmidt *et al.* (1962).

Analytical procedures. DNA was determined by a modification of the diphenylamine procedure (Burton, 1956) and by a fluorescent method using ethidium bromide (Prasad *et al.*, 1972). The orcinol procedure (Ogur & Rosen, 1950) was used to measure RNA; carbohydrate was estimated by the anthrone procedure (Johanson, 1953). Protein was determined by the Lowry method. Base composition of DNA was determined by hydrolysis with HClO_4 or formic acid followed by TLC. Quantitative measurements were made as described by Bendich (1957).

RESULTS

DNA content of xenosomes

Xenosomes were isolated and purified from *P. acutum* taken from both the mid-exponential (3 d) and early stationary (6 d) phases of growth, and analysed for DNA content. The DNA content of xenosomes isolated from the mid-exponential growth phase ($\pm$ S.D.) was significantly higher (8 ± 3 fg; six determinations of three preparations) than that of xenosomes taken from the early stationary phase (5 ± 1 fg; nine determinations of seven preparations). In a random population, cells are expected to contain from n to $2n$ the normal DNA complement in the form of replicating molecules, and in the early stationary growth phase it is expected that the genome consists of completed, non-replicating chromosomal DNA molecules. The analytical complexity of a genome containing 5 fg DNA and consisting of a single unique DNA sequence would equal 3.03×10^9 daltons.

Extraction of DNA

Symbiont and whole-cell DNA were examined chemically and spectrophotometrically to determine the degree of purity of the preparations. Less than 1% protein was present; carbohydrate was not detected. Chromatograms of HClO_4 and formic acid digests showed no evidence of a uracil spot. Absorbancy ratios (230/260 nm) averaged 0.42; 280/260 nm ratios averaged 0.51. These values are comparable to those reported for other DNAs (Marmur, 1961).

DNA from xenosome-free and xenosome-bearing protozoa, and from isolated and purified xenosomes, was subjected to preparative CsCl centrifugation (Fig. 1). A single DNA peak was observed with xenosome-free *P. acutum* (Fig. 1*a*), whereas a shoulder, representing DNA material of higher density than the main band, was seen in DNA of xenosome-bearing *P. acutum* (Fig. 1*b*). Figure 1(*c*) illustrates the CsCl profile of pooled fractions of the shoulder. Figure 1(*d*) represents DNA isolated from purified xenosomes. This DNA was indistinguishable from DNA derived from the recycling of the denser band of the gradient illustrated in Fig. 1(*c*) (gradient not shown).

DNA base composition

Analytical CsCl gradient profiles of purified DNA from xenosome-bearing and xenosome-free *P. acutum*, and from isolated and purified xenosomes, showed that the density of the main band of *P. acutum* DNA was 1.680 g cm^{-3} . DNA from xenosomes had a density of 1.694 g cm^{-3} . A shoulder at density 1.694 g cm^{-3} , observed in xenosome-bearing *P. acutum* DNA, was due to the presence of xenosome DNA. Mitochondrial DNA was not detected in either whole-cell xenosome-free or whole-cell xenosome-bearing *P. acutum* preparations.

The thermal transition of DNA isolated from xenosome-free *P. acutum* was fairly sharp and exhibited a uniform Gaussian distribution about the mean (Fig. 2*a*), while the thermal transition of DNA isolated from xenosome-bearing *P. acutum* exhibited a bimodal distribution about the mean (Fig. 2*b*). The thermal transition of DNA purified from xenosomes was very sharp; the Gaussian distribution about the mean was virtually unimodal and uniform (Fig. 2*c*). The values for the mid-point of thermal transition (T_m) obtained from *P. acutum* (symbiont-free)

Fig. 1. Preparative CsCl gradient centrifugation. (a) Whole-cell *P. acutum* (xenosome-free) DNA; (b) whole-cell *P. acutum* (xenosome-bearing) DNA; (c) pooled fractions from shoulder (tubes 40–60) of gradient in (b); (d) xenosome DNA. The DNA was labelled by culturing *P. acutum* (xenosome-bearing and xenosome-free) in the presence of [^{14}C]adenine. ---, CsCl density.

Table 1. *Base composition of whole-cell Parauronema acutum (symbiont-free) and purified xenosome DNA*

Values represent the means of duplicate analyses of three preparations, $\pm$ S.D.

Component	DNA	Composition (mol %)	
		Whole-cell <i>P. acutum</i>	Xenosomes
Guanine		10.3 $\pm$ 0.2	17.2 $\pm$ 0.4
Cytosine		9.9 $\pm$ 0.2	17.0 $\pm$ 1.2
Adenine		39.6 $\pm$ 0.3	33.2 $\pm$ 0.6
Thymine		40.2 $\pm$ 0.3	32.6 $\pm$ 1.1
G + C (chromatography)		20.2	34.2
G + C (T_m)		20.0	33.9
G + C (buoyant density)		20.4	34.7

Fig. 2. Thermal transition in SSC. (a) Whole-cell *P. acutum* (xenosome-free) DNA; (b) whole-cell *P. acutum* (xenosome-bearing) DNA; (c) xenosome DNA. ○, Hyperchromicity at 260 nm; ■, increments (A_{260} units).

Table 2. *Genome size from kinetic data*

Values represent the means of six determinations of three preparations $\pm$ S.D.

Method	Reassociation solution	$10^{-9} \times$ Molecular complexity (daltons)	
		T_4	Xenosome
Optical	1 M-NaCl	0.13 ± 0.11	0.35 ± 0.02
Optical	1 $\times$ SSC	0.13 ± 0.12	0.32 ± 0.04
Hydroxyapatite chromatography	0.12 M- NaH_2PO_4	0.13 ± 0.10	0.36 ± 0.03

and xenosome DNA were 77.5 °C and 83.2 °C, respectively. The base compositions of *P. acutum* and xenosome DNA calculated from these values are in excellent agreement with those derived from chromatographic and buoyant density data (Table 1). The results suggest that symbiont and protozoan DNA are native double-stranded molecules and are relatively homogeneous with respect to G + C content. It is unlikely that they contain significant amounts of unusual sugars or bases.

Genome size

Renaturation rate plots followed theoretical second-order kinetics for about 30% of the reaction when determined by the optical method. For this reason the renaturation rate constants were estimated from the initial renaturation rates. On the other hand, renaturation using hydroxyapatite chromatography closely followed second-order kinetics. The results (Table 2) show that the kinetic complexity determined by three procedures was comparable.

Fig. 3. Sedimentation pattern in a sucrose gradient of ^{14}C -labelled DNA gently released from xenosomes (○), co-sedimented with ^3H -labelled T_4 bacteriophage DNA (■).

Fig. 4. Effect of DNA concentration on sedimentation coefficient.

Thus, the size of the symbiont DNA genome, determined kinetically, was about 0.34×10^9 daltons. On the other hand, the size of the genome determined chemically was 3.03×10^9 daltons. These results suggest that the DNA genome of the symbiont contains nine copies of a unique sequence. What then is the arrangement of these unique sequences within the symbiont genome? Two possibilities suggest themselves: (1) the unique sequences are arranged in tandem on a single DNA molecule of about 3×10^9 daltons, or (2) the unique sequences are arranged within the symbiont as a collection of nine individual DNA molecules each of about 0.34×10^9 daltons. Since it had been shown through sedimentation rate measurements that gentle lysates of bacteria produce large numbers of intact genomes (Kavenhoff, 1972), we applied this procedure to measure the size of the symbiont genome. In order to obtain more accurate measurements of the sedimentation coefficient(s), bacteriophage T_4 labelled with $[^3\text{H}]$ thymidine was co-lysed with the symbionts in each run. Figure 3 illustrates a typical run. The rapidly sedimenting radioactive peak was identified as symbiont DNA by virtue of the fact that it was destroyed by the action of deoxyribonuclease. In a study of the size of the DNA genome of *Bacillus subtilis*, Kavenhoff (1972) found a strong dependence of the observed sedimentation rates on the speed of centrifugation. Our results with symbiont DNA showed only a negligible effect. The amount of DNA present significantly affected the s value, however (Fig. 4). The mean value ($\pm$ S.D.) for the sedimentation coefficient for 17 symbiont preparations (corrected for concentration and speed of centrifugation) was $85.7 \pm 9\text{S}$. From these data we calculate a molecular weight of $0.42 \pm 0.13 \times 10^9$ for the symbiont genome, assuming a linearly-permuted molecule. In the case of phage DNA, Hershey *et al.* (1963) have calculated that s (circular) = $s \times 1.13$ (linear). If we assume a circularly-permuted molecule, the genome size of the symbiont DNA would be $0.30 \times 10^9 \pm 0.09 \times 10^9$ daltons.

Table 3. Size, T_m , kinetic and analytical complexities of the DNA of symbionts of ciliates

Protozoan host	Stock	Symbiont	Size (width $\times$ length, μm)	T_m ($^{\circ}\text{C}$)	10 ⁻⁹ $\times$ Complexity (daltons)		No. of genomes	Reference
					Kinetic	Analytic		
<i>Parauronema acutum</i>	110-3	Xenosome	0.3 $\times$ 1.0	33.9	0.34	3.03	9	This study
<i>Paramecium octaurelia</i>	299	Lambda	0.65 $\times$ 3.18	26.0	0.39	7.5	19	Soldo & Godoy (1973)
<i>Paramecium octaurelia</i>	138	Mu	0.42 $\times$ 1.63	34.2	0.56	4.45	8	Soldo & Godoy (1974)
<i>Paramecium tetraurelia</i>	139	Pi	0.40 $\times$ 1.52	35.6	0.59	5.05	8-9	Soldo & Godoy (1974)
<i>Euplotes aediculatus</i>	—	Omikron	0.3 $\times$ 5.0	47.5	0.5	3.5	7	Schmidt & Heckmann (1980)

DISCUSSION

From the data presented in this paper, it is possible to exclude the model of the symbiont genome as a single molecule consisting of tandemly arranged DNA sequences. Clearly, the symbiont genome is composed of nine individual DNA molecules. Unfortunately, the data do not permit a clear-cut distinction between the linear or circular form. The size of the genome determined from kinetic data, 0.34×10^9 daltons, is intermediate between values of 0.30×10^9 (circular) and 0.42×10^9 (linear). It is possible that DNA released by gentle lysis of the symbionts sedimented as a collection of both circular and linear molecules and that the linear molecules arose through breakage during handling. An interpretation that the molecules are circularly permuted is supported in part by the fact that the value of 0.34×10^9 (from kinetic data) more closely agrees with the value of 0.3×10^9 (circular) obtained from sedimentation rate data. Attempts have been made to resolve this issue by examining, by electron microscopy, contour lengths of DNA molecules released from the symbionts by gentle lysis procedures, but they have been inconclusive. The largest intact molecule we have observed using these methods was linear and had a contour length of 160 nm, corresponding to a molecular weight of 0.32×10^9 . Nevertheless, a reasonable model for the structure of the symbiont genome is one in which the DNA exists in the form of nine circularly permuted, double-stranded molecules of unique sequence, each of molecular weight 0.34×10^9 .

Small size and multicopy genomes appear to be the rule for bacterial endosymbionts of ciliated protozoa. Table 3 summarizes the data on the genome structure of symbionts of ciliates for which detailed information is available. Although the data must be considered preliminary, some comments appear justified. First, the symbionts vary significantly in size and T_m values, suggesting a diversity of forms. With respect to size, values range from $0.3 \times 1.0 \mu\text{m}$ (xenosomes) to $0.65 \times 3.18 \mu\text{m}$ (lambda), corresponding to a difference in volume of almost 15-fold. Despite this comparatively large difference, the genome sizes of the symbionts, measured kinetically, fall within a relatively narrow range (0.34×10^9 daltons for xenosomes to 0.59×10^9 daltons for pi). These sizes are comparable to those found among the rickettsias (Bak *et al.*, 1969) and mycoplasmas (Bode & Morowitz, 1967), and are the smallest known for bacteria. If we assume an average protein (mol. wt = 45 000) is coded by a nucleotide sequence of about 8.2×10^5 daltons, the symbionts code for 400–700 proteins. By comparison, the free-living bacterium *E. coli*, which has a molecular complexity of 2.5×10^9 daltons (Cairns, 1963), codes for about 3000 proteins (Bode & Morowitz, 1967). Bacteriophage T₄ contains sufficient DNA to code for about 160 proteins. Gibson *et al.* (1971) reported values of 3.3×10^9 and 3.4×10^9 daltons for the kinetic complexities of mu endosymbionts of *Paramecium aurelia* stocks 551 and 540, respectively. Because their paper did not contain details of the conditions of their experiments it is not possible to interpret their results meaningfully. A report on bipolar bodies, bacterial endosymbionts of the flagellate *Crithidia oncopelti* (Spencer & Cross, 1975), suggests that the genome size of this endosymbiont, determined kinetically, was 2.7×10^9 daltons, equivalent in

size to that of *E. coli*. Tuan & Chang (1975) reported a very high value of 6.7×10^9 daltons for the kinetic complexity of the DNA of another bipolar body (*Blastocrothidia culicis*). In contrast, Schwemmler *et al.* (1975) found the kinetic complexity of leaf-hopper endosymbionts to range from 0.022×10^9 to 0.028×10^9 daltons, sufficient to code for only 25–30 proteins.

We view the small genome size of symbionts of ciliated protozoa as the result of loss of genetic material through selective mutational events involving deletions of portions of once larger genomes of free-living bacteria. In effect, these symbionts may represent an heterogeneous group of extant forms of ancient bacteria. Similar views have already been presented (Preer *et al.*, 1974; Soldo & Godoy, 1973). Evolution of multicopy genomes is looked upon in a similar light, i.e., adaptation to an intracellular environment. Multiple copies of specific gene sequences may serve as a 'hedge' against loss of symbionts through mutation. In this respect it is of interest to note that free-living and parasitic bacteria have mainly single-copy genomes (Bak *et al.*, 1970). The only exception reported thus far is that of *Azotobacter vinelandii*, which has a multicopy genome (Sadoff *et al.*, 1979). Multicopy genomes are common among chloroplasts and mitochondria (Gillham, 1978).

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