*PARTIAL PURIFICATION OF NATIVE rRNA AND tRNA CISTRONS FROM MYCOPLASMA SP. (KID)**

By John L. Ryan and Harold J. Morowitz

DEPARTMENT OF MOLECULAR BIOPHYSICS, YALE UNIVERSITY

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Abstract.—Precise optical melting profiles of purified DNA from *Mycoplasma* sp. (Kid) show a secondary hyperchromic rise, corresponding to 1.4 per cent of the total DNA, occurring at 88°C, while the bulk of the DNA melts at 79.5°C, indicating an average base composition of 24.9 per cent guanine-cytosine (G-C). A method is presented, using sonication followed by hydroxyapatite column chromatography, for the partial purification of regions of a genome which contain significantly higher G-C than the average value for the genome. The procedure does not involve denaturation and renaturation of the high G-C material so that purified DNA is in its native, double-stranded state and has a normal melting profile. When applied to *Mycoplasma* sp. (Kid), the method yielded a fraction of native DNA enriched 40 times with respect to those regions coding for rRNA and tRNA. This enriched DNA has a saturation hybridization value of 15.9 per cent with Kid rRNA plus tRNA. The saturation hybridization values of the bulk DNA with rRNA and tRNA are 0.26 per cent and 0.16 per cent, respectively. Based on a genome size of 6.84×10^8, obtained by electron microscopy, this indicates that *Mycoplasma* sp. (Kid) contains only enough ribosomal DNA to code for one set of 23*S* plus 16*S* rRNA and only enough DNA complementary to tRNA to code for 44 different tRNA molecules.

Among the species of procaryotic cells that have been examined, the mean guanine-cytosine (G-C) content of DNA varies between 25 per cent and 75 per cent.[1] The content of these bases in bacterial rRNA and tRNA varies between 45 per cent and 65 per cent,[2, 3] a much smaller range than that observed for DNA. Thus, one would expect a compositional heterogeneity in all low G-C genomes between those regions coding for rRNA and the major portion of the DNA which codes for messenger RNA.

Differential melting is one of the techniques which can be used to distinguish sequences of DNA bases which vary with respect to G-C content.[1] In this paper the use of differential melting for the partial purification of a chemically distinct region of the chromosome of *Mycoplasma* sp. (Kid) is described. This region, which melts at a high temperature, contains DNA complementary to both tRNA and rRNA.

Mycoplasma sp. (Kid) was chosen for these experiments due to the extremely low G-C content of its DNA and the small size of its genome. Kid DNA is among the lowest G-C DNA's (25%) yet reported for microorganisms.[1, 4]

Materials and Methods.—(a) *Bacterial strains: Mycoplasma* sp. (Kid) was used in all experiments. Control DNA and RNA were prepared from *Escherichia coli* B.

(b) *Growth of cells: Mycoplasma* sp. (Kid) was routinely grown in medium composed of 2% Difco Tryptose, 0.5% NaCl, 0.4% tris(hydroxymethyl)aminomethane, 1.0%

glucose, 1.0% Difco PPLO serum fraction, and 150,000 units/liter penicillin G (Eli Lilly & Co.). Cells were grown statically at 37°C.

For P^{32} incorporation, the tryptose concentration of the above medium was decreased to 0.5% and 10 mc of carrier-free $H_3P^{32}O_4$ in water (New England Nuclear, Boston, Mass.) were added per two liters of medium.

For H^3 incorporation, the tryptose concentration was decreased to 1.0% and 1.0 mc of thymidine methyl-H^3, 15 c/mM (New England Nuclear, Boston, Mass.), was added per two liters of medium.

E. coli B was routinely grown in 0.8% Difco nutrient broth at 37°C with aeration. For P^{32} incorporation into *E. coli*, P minimal medium[5] was used with the addition of 1.0 mc/liter carrier-free phosphate as above. For C^{14} incorporation into *E. coli*, C minimal medium[5] was used with 0.1% glucose and 250 μc/liter of uniformly labeled glucose, 150 μc/mM (New England Nuclear, Boston, Mass.).

(c) *DNA isolation:* DNA was isolated from late log phase cells by the method of Marmur[6] with a pronase digestion step included after ribonuclease treatment.[7] An additional step included was refrigeration for at least 12 hr at 4°C of the chloroform mixture after the first deproteinization. This facilitated the deproteinization and improved the recovery of DNA.

(d) *rRNA isolation:* rRNA was isolated from late log phase cells by lysis in a French pressure cell, followed by ribosome purification and phenol extraction as described by Kirk and Morowitz.[8] It was further purified by repeated deoxyribonuclease treatments and chromatography on methylated albumin kieselguhr (MAK).[7]

(e) *tRNA isolation:* tRNA was isolated from log phase cells by phenol extraction and chromatography on cellulose diethylaminoethyl ether (DEAE) as described by Hayashi *et al.*[9]

(f) *DNA melting:* DNA was melted in a modified Beckman DU spectrophotometer. Three thermal spacers were inserted on each side of the cuvette chamber. Water was circulated through the interior two from a thermostatically controlled Haake water bath. Tap water was circulated through the outside spacers to keep the phototube housing cool during the heating. Teflon-stoppered spectrophotometer cells (3-ml capacity) were used in all melting experiments. It was necessary to use cells with tapered stoppers in order to maintain optimum control over evaporation at elevated temperatures. At least 15 min were allowed for thermal equilibrium after each temperature was reached. Under these conditions no measurable evaporation took place at 96°C for times up to 1 hr. Temperature within the cuvette chamber was continuously monitored during melting by inserting a thermometer into a cuvette filled with water. The thermometer was graduated in 0.2°C steps between 48°C and 102°C.

(g) *Sonication of DNA:* Sonication of DNA was performed with a Branson model W140D sonifier using a $^1/_2$-in. taped probe at full intensity. Output was 85 w. DNA was dissolved in 0.15 *M* NaCl, 0.015 *M* Na citrate, pH 7.0 ± 0.1 (SSC). The concentration was kept at approximately 20 μg/ml. The solution was bubbled with N_2 before and during the sonication. Sonication was carried out for two 2-min intervals in a rosette cooling flask chilled in an ice bath. The sonication procedure usually produced a small (2–3%) rise in the optical density measured at 260 mμ. Therefore, DNA was always purified by MAK column chromatography after sonication. Sonic fragments from this procedure have a sedimentation coefficient in the analytical ultracentrifuge (S_w^{20}) of 6S when centrifuged in alkaline NaCl. This corresponds to a molecular weight of 150,000[10] for the single-stranded fragments. The molecular weight of the sonic fragments was also measured directly with the electron microscope (EMU 3B, Radio Corporation of America) using the technique of Bode and Morowitz.[11] This resulted in a value of 250,000 ± 10% for the double-stranded fragments. Thus, the sonication procedure used did not produce significant single-strand breaks in the DNA.

(h) *Chromatography:* Hydroxyapatite chromatography was carried out as described by Miyazawa and Thomas.[12] The column consisted of about 1 ml (packed vol) of hydroxyapatite. Approximately 200 μg of DNA were bound per column. Columns were eluted

with either a stepwise gradient of phosphate buffer, pH 6.8 (equal concentrations of Na_2HPO_4 and NaH_2PO_4), or washed with 0.17 *M* phosphate buffer as a function of temperature. Enrichment of Kid DNA with respect to high G-C regions was carried out by differential melting while adsorbed to hydroxyapatite. In a routine enrichment, about 1.0 mg Kid DNA was sonicated, purified on an MAK column, dialyzed into 0.01 *M* phosphate buffer, and loaded on a hydroxyapatite column. The column was washed with 0.17 *M* phosphate buffer to remove any unadsorbed native DNA and any residual single-stranded DNA. The temperature of the column was raised to 70°C and the column was washed again with an excess (10 ml) of 0.17 *M* phosphate buffer. A Haake thermostatically controlled water bath was used to maintain temperature in the column to within 0.1°C. Temperature was measured with a YSI model 42SC telethermometer using a Teflon-covered thermistor probe. The temperature of the column was then raised to about 86.0° to melt all low G-C DNA and denatured DNA was eluted with 20 ml 0.17 *M* phosphate buffer. A subsequent 5-ml elution with 0.27 *M* phosphate buffer served to remove remaining native DNA from the column. The native fractions from hydroxyapatite columns were pooled and rechromatographed on an MAK column for purposes of concentration and to remove some ultraviolet-absorbing material that resulted from the hydroxyapatite chromatography. This fraction was designated "enriched" or high G-C DNA.

MAK chromatography was carried out as described by Gurney.[13] Only one layer of protein-coated kieselguhr was used in column preparation. The capacity of a column with a diameter of 2 cm was about 750 μg native DNA. In all experiments, DNA was routinely purified by MAK chromatography after sonication and after hydroxyapatite chromatography.

(i) *Base ratios of rRNA:* The nucleotide composition of Kid rRNA was determined by alkaline hydrolysis and chromatographic separation using Bio-Rad AG 1-X8, formate form, 200–400 mesh, following the procedure of Kirk and Morowitz.[8] P^{32} *E. coli* rRNA was cohydrolyzed and used as an internal control.

(j) *Paper electrophoresis:* The base ratios of unenriched and enriched DNA were determined by paper electrophoresis of nucleotides. The digestion of the DNA to nucleotides was accomplished by sequential enzymatic hydrolysis using the procedure of Miyazawa and Thomas.[12] Electrophoresis was carried out for 1 hr at 100 v/cm in an apparatus similar to that described by Smith.[14, 15] P^{32} Kid DNA was cohydrolyzed and run with C^{14} *E. coli* DNA as an internal control. Strips were dried and counted in a Vanguard strip counter.

(k) *Hybridization:* DNA-RNA hybridization was carried out on nitrocellulose filters[7] (Schleicher and Schuell, type B-6).[7] Fixation of the DNA on filters was done in 6 × SSC, and all subsequent steps were carried out using 2 × SSC. This procedure tended to maximize DNA adsorption and minimize RNA background. Hybridization mixtures were incubated for 12–15 hr at 66°C. An excess of cold rRNA was added in all tRNA hybridization experiments to effectively compete with contaminating labeled rRNA breakdown products present in the tRNA preparation. H^3 DNA and P^{32} RNA were used in all hybridization experiments. Counting was done in a Packard Tri-Carb liquid scintillation counter.

In order to determine accurate saturation hybridization values, the molecular weight of the genome must be known. The molecular weight of the genome of *Mycoplasma* sp. (Kid) has been determined to be $6.84 \times 10^8 \pm 10\%$ by direct electron microscopic measurement.[11] In this method the cells are lysed by dialyzing sodium dodecyl sulfate into them in the presence of sucrose and pancreatic lipase (Worthington Biochemical Corp.). The lysate is then spread on the surface of water in a protein film and samples are picked off the surface using electron microscope grids. Only complete circles, containing no "centers" in the display, were used to make the measurements.

The amount of RNA bound in a saturation hybridization experiment is equivalent to the amount of single-stranded DNA complementary to it. It has been shown by a number of laboratories[16–20] that only a single strand of native DNA is used as a primer for

RNA synthesis both *in vivo* and *in vitro*. Thus, the amount of native DNA involved in the production of a molecule of RNA may be twice the weight of the transcribed RNA.

Results.—(a) *Melting of Kid DNA:* It has been shown that the melting temperature of DNA is proportional to the G-C content of the DNA as expressed by the following relationship; $T_m = 69.3 + 0.41$ (G-C).[1] When Kid DNA was melted in SSC (Fig. 1), a shoulder was observed on the curve. Most of the melting occurred at 79.5°C, indicating a base composition of 24.9% ± 0.3% G-C. The second hyperchromic rise, occurring at 88°C, is of particular interest. It indicates that there is a small amount, approximately 1.4 per cent, of the DNA in the preparation which has a base composition of about 46 per cent G-C. The same phenomenon results when sonicated Kid DNA is melted (Fig. 1).

The base composition of the bulk DNA has been corroborated by cesium chloride equilibrium banding in the model E analytical ultracentrifuge. With *E. coli* DNA as a reference marker, the density of Kid DNA was calculated to be 1.684 gm/c^3. This corresponds to a base composition of 24.5% G-C. A satellite band corresponding to the secondary rise was not observed. This is most likely due to the fact that the high G-C DNA is dispersed throughout the chromosome.

(b) *Nucleotide analysis of Kid rRNA and tRNA:* The results of nucleotide analysis of Kid rRNA and tRNA are shown in Table 1. They demonstrate that Kid DNA should contain a region or regions having a high G-C compared with the average value of 24.9 per cent.

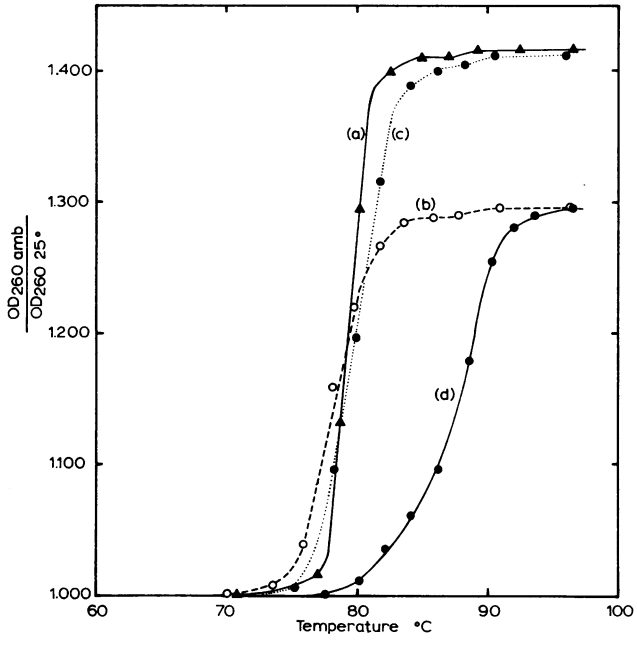

FIG. 1.—(*a*) Melting profile of unsonicated Kid DNA in SSC. (*b*) Melting profile of sonicated Kid DNA in SSC. (*c*) Melting profile of sonicated Kid DNA in 0.17 *M* phosphate buffer, pH 6.8. (*d*) Melting profile of enriched Kid DNA in 0.17 *M* phosphate buffer. Curve *d* corresponds to a partial purification of the small shoulder visible in curves *a*, *b*, and *c* at about 88°C.

TABLE 1. *Nucleotide analyses of Mycoplasma sp. (Kid) tRNA, rRNA, and bulk DNA.*

Sample	A	G	C	U (T)
Bulk DNA	37.5%	12.5%	12.5%	(37.5%)
rRNA	28.0 ± 1.0%	26.8 ± 1.0%	21.1 ± 1.0%	24.1 ± 1.0%
tRNA	22.0%	29.2%	24.6%	24.3%

(c) *Hydroxyapatite chromatography:* The elution pattern of both native and denatured sonicated DNA as a function of buffer concentration from hydroxyapatite is found in Figure 2. These experiments were done using both C^{14} *E. coli* DNA and P^{32} Kid DNA. From these results it is apparent that 0.17 *M* phosphate buffer serves to elute all denatured DNA while native DNA remains adsorbed to the column. In order to elute native DNA, it is necessary to increase the molarity of the phosphate buffer to 0.27.

A thermal elution profile for sonicated Kid DNA from hydroxyapatite is given in Figure 3. The T_m of 81°C corresponds to the melting temperature observed in optical melting when the solvent used is 0.17 *M* phosphate buffer (Fig. 1). Figure 3 reveals that there is a small fraction of DNA (approximately 1.0%) melting above 89°C. This fraction could account for the 1.4 per cent secondary rise observed in the optical melting curves. It was collected as described in *Materials and Methods.*

(d) *Base ratios of enriched DNA:* Paper electrophoresis of nucleotides was carried out on enzymatic digests of enriched P^{32} Kid DNA. The results of four

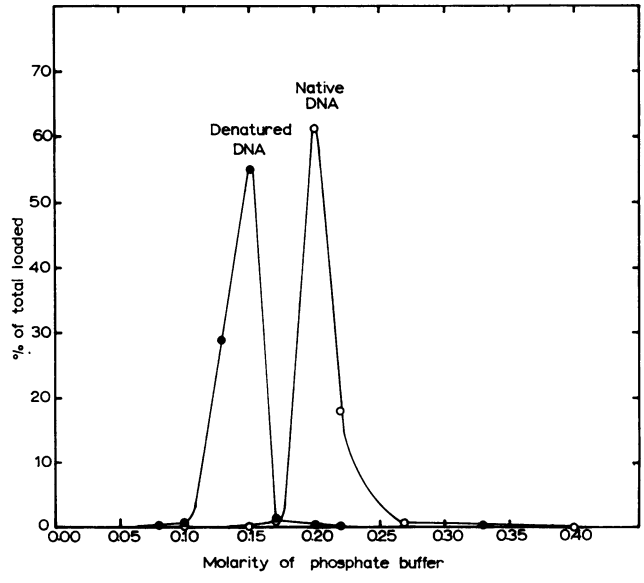

FIG. 2.—Separation of native and denatured DNA by chromatography on hydroxyapatite. Recovery from hydroxyapatite columns was nearly 100% for both native and denatured DNA. The column was washed with three 5-ml vol of buffer at each molarity. This served to fully elute any DNA freed by the buffer. It is apparent that 0.17 *M* phosphate buffer will serve to seperate native from denatured DNA.

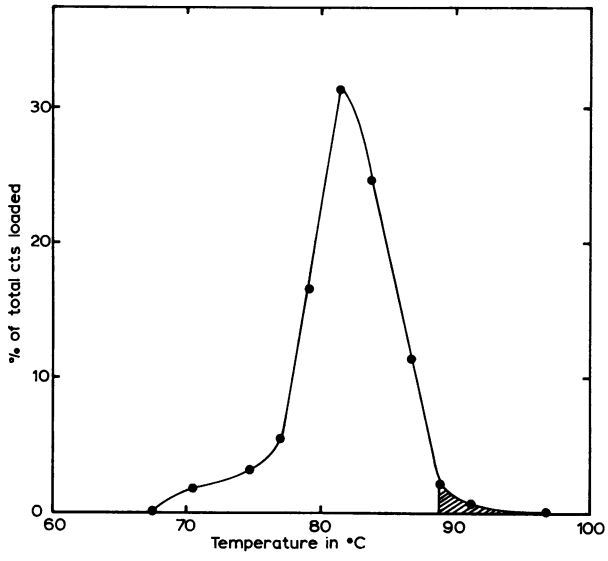

FIG. 3.—Thermal elution profile of Kid DNA from hydroxyapatite using 0.17 *M* phosphate buffer and eluting as a function of temperature. As the sonic fragments melt, they are eluted by the buffer. The shaded area represents that DNA which was left native during an enrichment and eluted with higher-molarity (0.27 *M*) buffer.

separate experiments show that the enriched DNA contains 38.0% ± 1.0% G-C. Based on the assumption that all high G-C DNA within the Kid genome contains 46% G-C, and that before enrichment this fraction amounts to 1.4 per cent of the total DNA, these results indicate that approximately a 44× enrichment of the high G-C DNA has been effected.

Optical melting was also carried out on the enriched fraction. Melting of more of the same sample which had been subjected to enzymatic hydrolysis corroborated the 44× enrichment value. Later enrichments, however, have yielded DNA which appears to be much more pure than earlier samples in that most of the low G-C DNA is absent. A melting curve of this material is shown in Figure 1. It appears from this curve that almost pure high G-C DNA can be isolated from *Mycoplasma* sp. (Kid).

(e) *Hybridization:* DNA-RNA hybridization was carried out with enriched and unenriched *Mycoplasma* DNA and rRNA and tRNA. The saturation hybridization value for rRNA and total Kid DNA was 0.26 per cent. The saturation value for Kid tRNA and total Kid DNA was 0.16 per cent. The saturation curve for the tRNA hybridization is presented in Figure 4. Thus, on the basis of the rRNA and tRNA hybridization, the 1.4 per cent high G-C DNA observed in the optical melting is not completely accounted for (0.52% + 0.32%). When hybridization of Kid rRNA and tRNA with enriched DNA was carried out, the respective saturation values were 12.7 per cent and 3.2 per cent. Thus, approximately 32 per cent of the native enriched DNA is involved in coding for rRNA and tRNA (2 × 15.9%). This amounts to approximately a 50× enrichment of the rRNA coding regions and a 20× enrichment of the tRNA coding regions. The error involved in determining the saturation values for tRNA could easily account for the difference in the enrichment factor. Optical melting of the same sample of enriched DNA used in the hybridization experiments also

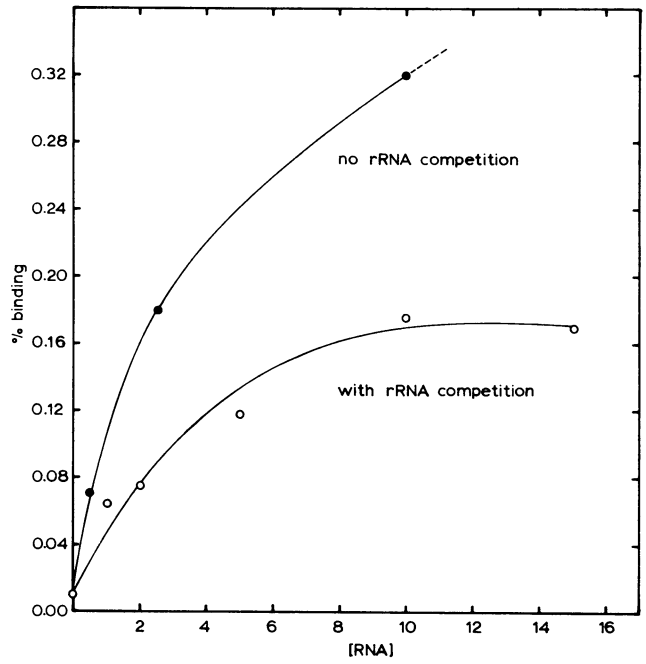

FIG. 4.—Saturation hybridization curve for Kid tRNA and bulk Kid DNA. It is apparent that it is necessary to compete with rRNA breakdown products using 10× the concentration of cold rRNA as a competitor. Only with this competition is a saturation plateau obtained. In most hybridization experiments 10 μg of H^3 DNA was fixed to the filters and incubated with various concentrations of P^{32} tRNA or rRNA.

indicated a 50× enrichment of the high G-C fraction of the DNA. From other melting results a greater enrichment of the rRNA and tRNA coding regions appears possible using this technique. The factor of enrichment is extremely dependent on very small changes (0.1°C) in the temperature of the hydroxyapatite column during chromatography at 86°C. Experiments are being carried out currently to achieve the maximum enrichment as measured by both hybridization and optical melting.

Based on a genome molecular weight of 6.84×10^8, the 0.26 per cent saturation hybridization value for rRNA indicates that there is enough DNA to make up one cistron each for 16*S* and 23*S* rRNA. This calculation is based on assigning a molecular weight of 1.1×10^6 to 23*S* rRNA and 0.56×10^6 to 16*S* rRNA.[21] The 0.16 per cent saturation hybridization value for tRNA indicates that there is enough DNA to code for a least 44 different tRNA molecules. This calculation is based on an average molecular weight of 25,000 daltons for tRNA.

Discussion.—The technique described for partial purification of native rRNA and tRNA cistrons is applicable to any species which has a DNA base composition which is significantly lower in G-C than the rRNA and tRNA. The outstanding feature of the technique as compared to other techniques for isolation of ribosomal cistrons[22] is that the cistrons involved are never denatured or renatured in the course of the procedure. The fact that the hyperchromicity of the enriched

DNA is less (see Fig. 1) than that of unenriched sonicated DNA seems to be due to the presence of ultraviolet-absorbing material resulting from the hydroxyapatite chromatography.

The sonication step was necessary to effectively free the high G-C regions from large amounts of contaminating low G-C DNA. The molecular weight of the bulk DNA was about 2×10^7 daltons as measured by sedimentation in the analytical ultracentrifuge.[10] When hydroxyapatite chromatography was carried out of this DNA, no high G-C DNA was observed in the thermal elution profile. As the DNA was sheared to progressively lower molecular weights, a small high G-C fraction began to be enriched as evidenced by optical melting. It was found that maximum enrichment was achieved by reducing the molecular weight as much as possible. Thus, the sonication step was incorporated into the enrichment procedure.

Mycoplasma sp. (Kid) has a genome about one-fourth the size of that of *E. coli*, yet it contains 40 per cent as much genetic material for the production of rRNA. Kohne[22] has shown that *E. coli* has enough ribosomal DNA to account for five sets of 16*S* and 23*S* rRNA. Using reassociation kinetics, he has also shown that at least four of these five sets are similar. Since *Mycoplasma* sp. (Kid) contains only enough DNA complementary to rRNA to produce one set of 16*S* and 23*S* rRNA, it appears that only one species of each type of rRNA is required for the functioning of this organism.

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