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R-Bodies in Newly Isolated Free-Living Hydrogen-Oxidizing Bacteria

J. Lalucat[1], O. Meyer[2], F. Mayer[2], R. Parés[1], and H. G. Schlegel[2]*

[1] Departamento de Microbiologia, Facultad de Biología, Universidad de Barcelona, Pza. Universidad, Barcelona, Spain
[2] Institut für Mikrobiologie der Universität Göttingen, Grisebachstrasse 8, D-3400 Göttingen, Federal Republic of Germany

Abstract. R-Bodies have been found in a recently isolated pseudomonas-like free-living hydrogen oxidizing bacterium. Their isolation, fine structure and chemical composition are described and compared with the R-bodies from the kappa particles (*Caedobacter*), obligate endosymbionts of *Paramecium aurelia*. The 2K1 R-bodies exhibited essential characteristics of the kappa R-bodies; however, their size and some other structural aspects proved that they represent a new type of R-bodies. The presence of phage tail-like particles in cells induced with Mitomycin C is in favour of the hypothesis that the R-bodies might be coded by defective prophages, or by extrachromosomal elements.

Key words: R-Bodies – Kappa particles – Free-living hydrogen bacteria – Induction – Electron microscopy – Chemical composition – Defective prophages – Plasmids.

The refractile bodies (R-bodies) are inner structures of the kappa particles (*Caedobacter*), bacterial endosymbionts which are present in many stocks of the *Paramecium aurelia* species complex. The kappa particles, in contrast to strain 2K1, are obligate endosymbionts, and cannot be cultivated without their host. Their presence in the paramecium confer to it the killer character. In its compact form the R-body is a proteinaceous ribbon wound into a tight roll consisting of about 10 turns, but it can also unroll into a long twisted filament (Preer et al., 1974). The R-bodies are presumably coded by a defective prophage, whose spontaneous induction determines, in a lethal synthesis, the production of defective phages and R-bodies (Dilts, 1977). Preer suggests that the R-bodies play an important role in delivering the toxic substance in the sensitive paramecia, but it is not yet precisely known how this might be achieved.

* To whom offprint requests should be sent

In a newly isolated free-living hydrogen oxidizing bacterium (strain 2K1) we have described "spiral bodies", intracytoplasmic membraneous structures rolled up like a dialysis tube (Lalucat and Mayer, 1978). Spiral bodies could be observed in all growth phases, in cells grown autotrophically and heterotrophically. The fine structural organization of these spiral bodies, as principally studied in ultrathin sections (Lalucat and Mayer, 1978), is further analyzed by detailed electron microscopical studies described in the present paper. In addition, an isolation procedure for these particles is described, together with results on their chemical composition and induction experiments with Mitomycin C and UV treatment. Based on these findings, similarities between "spiral bodies" and "R-bodies" (refractile bodies) are evident. As the term "R-body" is introduced, it will also be used for the "spiral bodies" in cells of strain 2K1.

Materials and Methods

Organism. Bacterial strain 2K1 is a recently isolated free-living hydrogen oxidizing bacterium. It is a *Pseudomonas*-like bacterium, probably related with *Pseudomonas pseudoflava* (Auling et al., 1978).

Growth Media and Culture Conditions. The basal medium used for growth was the mineral medium described by Schlegel et al. (1961). 1 ml/l of trace elements solution SL6 of Pfennig (1974), 10-fold concentrated, was added. The mineral medium was supplemented for autotrophic growth with 0.05% $NaHCO_3$, and for heterotrophic growth with sucrose at 1% final concentration. The cells were cultivated autotrophically at 30° C in 1 l Erlenmeyer flasks with 100 ml mineral medium in desiccators under an atmosphere of 10% O_2, 10% CO_2 and 80% H_2 (with or without shaking), or in a 10 l fermenter (Braun-Biostat, Melsungen, FRG) with continuous flow of the same gas mixture at a rate of 500 ml/min, and stirred at 400–600 rev/min. For plates, the medium was solidified with 1.5% Difco Agar (final concentration).

Isolation of R-Bodies. Autotrophically grown cells were centrifuged, washed twice and resuspended in potassium phosphate buffer (50 mM, pH 7.0) at a concentration of 5 g wet weight/100 ml buffer. The suspension was treated 18 h with Na-dodecyl sulfate (SDS, 0.1%

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final concentration) at room temperature with stirring. DNase was added to diminish the viscosity of the lysate; after centrifugation (30 min at 4° C and 10,000 rev/min, rotor 8 × 90 Zeta), the pellet was homogenized and resuspended in 50 ml buffer (for 10 g original wet weight). The suspension was sonicated (2 min/ml, 60 W) in 3 ml fractions in 10 ml glass centrifuge tubes with a Braun Sonic Ultra Sound 300. The lysate was finally centrifuged 30 min at 20,000 rev/min (rotor 8 × 20, Omikron). The pellet was resuspended in 7 ml buffer with a potter homogenizer, and 2 ml were layered in a pre-formed linear sucrose gradient in water (55 – 80 %, weight/volume), and centrifuged for 33 h at 4° C and 35,000 rev/min (rotor 6 × 13 SW, Vacufuge). The centrifuge tube was punctured at the bottom, and the fractions were collected in a LKB collector (5 drops per fraction). In routine experiments, the fraction with the R-bodies was obtained from the top of the tubes with a syringe after removal of the upper fractions not containing R-bodies. The R-body fraction was diluted in buffer, centrifuged and resuspended. This suspension was filtered through a 0.1 μm pore size membrane filter, washed with 50 ml H_2O, and resuspended in 50 mM potassium phosphate buffer, pH 7.0.

Dry Weight Determination. 2 ml of the R-body suspension were filtered through a dried and weighted 0.1 μm pore size membrane filter (25 mm diameter, Sartorius Membranfilter, GmbH), washed with 20 ml H_2O and dried over night at 75° C.

Determination of Protein. 1 ml aliquots of the R-body suspension with 1 ml H_2SO_4 conc. p.a. and selenium catalyst were boiled for 2 h in Kjeldahl flasks and cooled. The digested samples were titrated with 2N KOH and filled up to 100 ml with water. The ammonium ion content was determined with Nessler reagent (2.5 ml titrated sample, 0.05 ml Seignette salt solution and 0.1 ml Nessler reagent). Standard curves were performed with bovine serum albumin and ammonium sulfate. The protein content was also estimated according to Hartree (1972) and according to Bradford (1976).

Carbohydrates were measured according to Trevelyan and Harrison (1952) with the anthrone method.

Lipids were determined with a colorimetric method (Boehringer Mannheim, cat. n. 124 303) based on the sulfophosphovanillin reaction.

Mytomycin C Treatment. The minimal inhibitory concentration was determined in cells growing in liquid surcrose medium in test tubes with different Mitomycin C concentrations (Serva, Heidelberg). Mitomycin C (0.1 mg/ml) was added to 10 ml cultures of cells growing exponentially in mineral medium supplemented with peptone (0.25 %), yeast extract (0.25 %) and Na-succinate (0.25 %). The incubation time was 1 h. The cells were then centrifuged, resuspended in the same growth medium, allowed to grow for 7 h, plated on agar medium mineral plates, and incubated under autotrophic conditions for a period of 14 days.

Ultraviolet Irradiation. Autotrophically grown cells in liquid culture were centrifuged and resuspended in buffer, diluted to 10^6 cells/ml and exposed to the UV irradiation of an Original-Hanau Germicidal Lamp (Hanau GmbH, FRG) at a distance of 40 cm; the death rate was determined. For possible prophage induction an irradiation time of 20 s was chosen, during which 99 % of the cells were inactivated. The irradiated cells were then incubated under autotrophic conditions.

Electron Microscopy. 4 % uranyl acetate (pH 4.8) was used for negative staining by the technique of Valentine et al. (1968). Electron micrographs were taken with a Philips EM 301 electron microscope at calibrated magnifications. Acceleration voltage was 80 kV.

Results

Number and Structure of the R-Bodies from Strain 2K1

With the negative staining technique we have estimated the ratio of cells containing R-bodies in samples taken from different growth phases, and in different culture conditions (solid and liquid media, with and without shaking, in autotrophic and heterotrophic conditions). In all cases we could find cells with R-bodies. The number of R-bodies was highest in samples taken from the stationary growth phase of autotrophic cultures without shaking. The fraction of cells with R-bodies varied from 1 – 43 %.

The number of R-bodies per cell was also variable. Frequently cells contained only one R-body, but in the stationary growth phase two or three R-bodies could be present within the cell. Cells irradiated with ultraviolet light could form long filaments, and in that case the number of R-bodies per cell could increase up to 10.

In negatively stained preparations, the R-bodies from strain 2K1 appeared as intracytoplasmic membranes rolled up in a spiral, with an average diameter of 0.25 μm (0.20 – 0.32) (Figs. 1 and 10). In exceptional cases, they could measure 0.5 μm. The number of turns varied from 3 to 13, but the diameter of R-bodies was always very similar. Each spiral turn was about 11 nm thick.

In side-on view (Figs. 2 – 4) the R-bodies were, on average, 0.21 μm (0.17 – 0.23) high. As the image in negatively stained samples is a projection from the whole particle the side-on view of an R-body looks like a section. The ribbon could become smaller when

All electron microscopial preparations were negatively stained with uranyl acetate. Dimensions are given in μm

Fig. 1. Autotrophic cell in the stationary growth phase with one R-body in face-on view. Inset: R-body in face-on view, high magnification

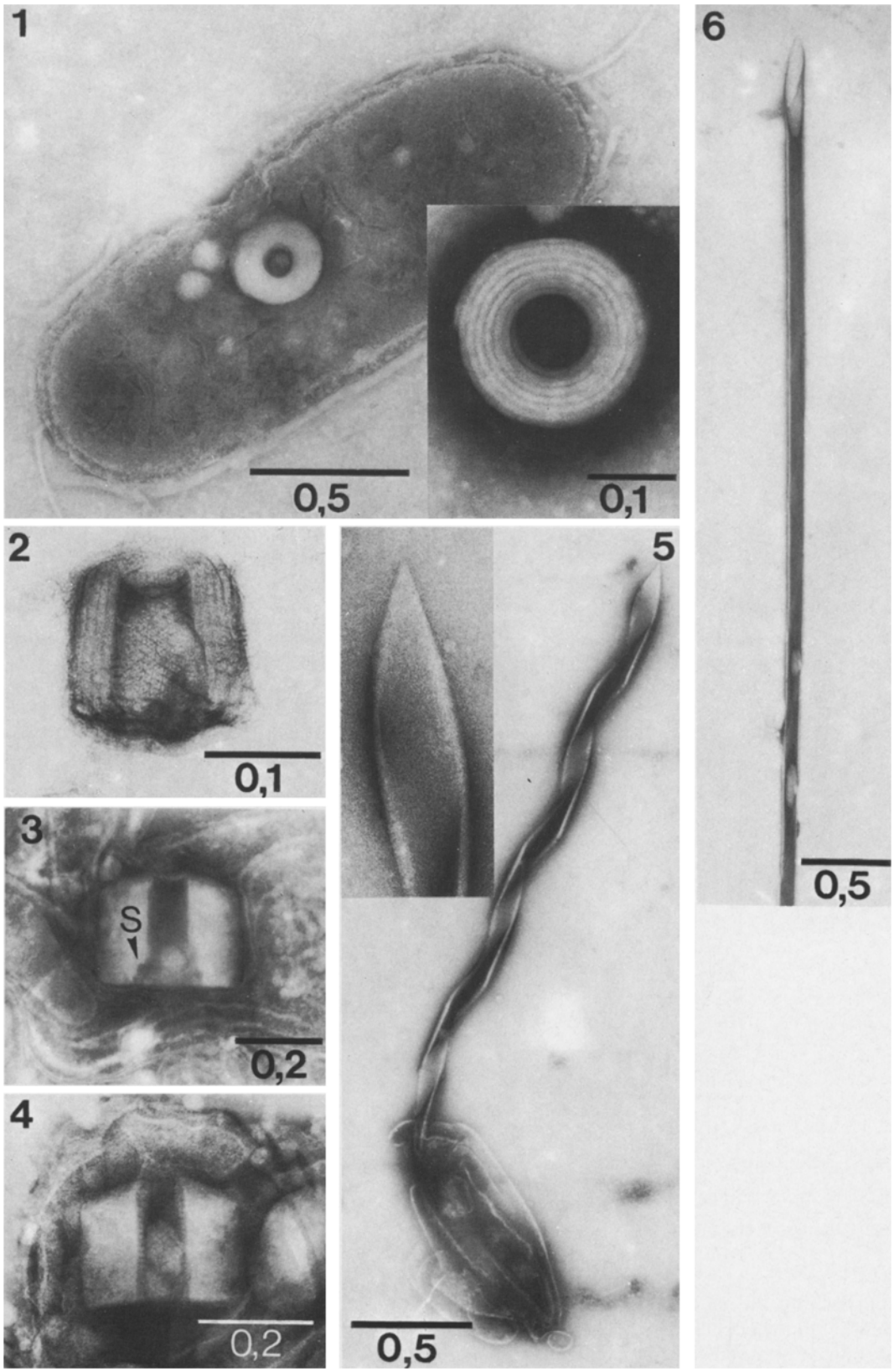
Fig. 2. R-body in side-on view, surrounded by membrane remnants. Inner side with a regular striation

Fig. 3. R-body in side-on view, surrounded by membrane remnants, with "steps" (*S*)

Fig. 4. R-body in side-on view, surrounded by membrane remnants. The inner side with a regular striation appearing like a furrow

Fig. 5. Unrolled R-body sticking out from a lysed cell. Acute end (inset) not revealing any structural subunits

Fig. 6. Isolated and unrolled R-body with a cannule aspect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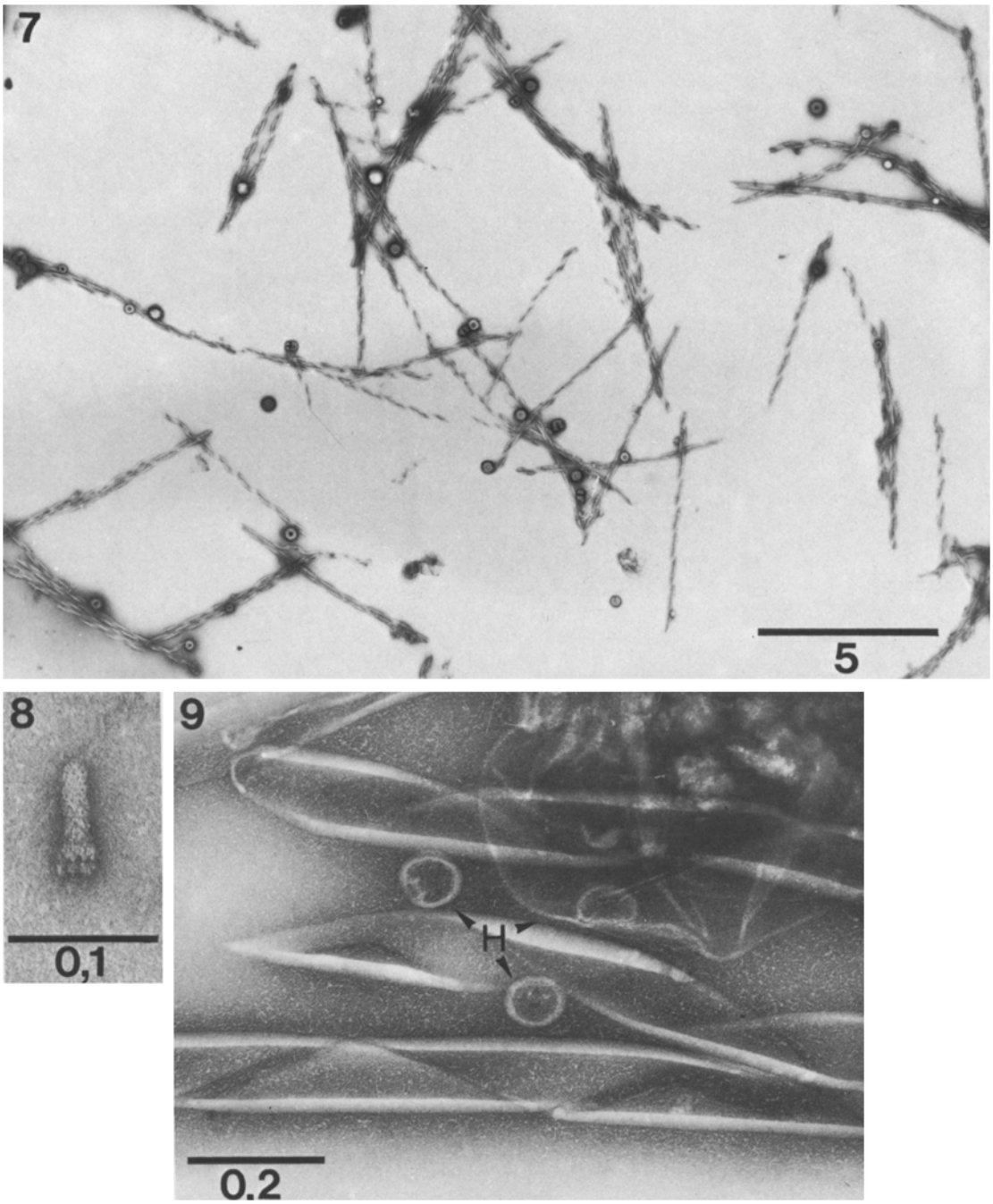

Fig. 7. Purified suspension of R-bodies in buffer

Fig. 8. Liberated bacteriophage tail-like structure only present in cells treated with Mitomycin C

Fig. 9. Unrolled R-bodies with bacteriophage head-like structures (*H*)

approaching the inner end, and R-bodies in side-on view could then exhibit "steps" (Fig. 3). The inner side of some R-bodies revealed a regular striation, forming rhombs, with their major diagonal about perpendicular to the R-body axis, and with angles of 112° and 68° (Figs. 2 and 4).

Spontaneously, or with Mitomycin C induction, the R-bodies could unroll (Figs. 5, 10 and 11), starting from the inside and forming a twisted filament with an acute (inner) and a blunt (outer) end. The unrolled R-bodies were, on average, 6 μm (3.5 – 7.2) long. If a filament was not completely loose, each turn overlapped the next

one, and the filament appeared like a canule with an acute bevelled end (Fig. 6). In the unrolled R-body, the regular striation mentioned above was no longer visible, and in negatively stained electron microscopic samples we could not detect any indication for structural subunits (Fig. 5, inset).

Isolation of R-Bodies

According to electron microscopical examination, treatment with sodium dodecylsulphate resulted in the disruption of all cells. However, the R-bodies were not affected; they maintained the same structure as in the cells. They were frequently enclosed, together with polyhydroxybutyrate granula, within membrane remnants, and during sucrose density gradient centrifugation, they accumulated in the same fraction. For separation of the R-bodies, the lysate was sonicated. Sonication for 2 min/ml at 60 W resulted in liberation of the R-bodies without affecting them; prolonged treatment (4 min) resulted in unrolling, and frequently fragmentation occurred.

The sucrose gradient centrifugations were performed for different periods (14, 24, 33, 48 h); the best results were obtained with runs lasting 33 h. The R-bodies sedimented in the 73% (weight/volume) sucrose fraction, which corresponds to 1.4064 g/cm^3. Approximately 60% of the R-bodies unrolled during the centrifugation. The fraction appeared to contain only R-bodies without contamination by other cell constituents (Fig. 7). From 11 g wet weight we could isolate 31.5 mg dry weight of purified R-bodies.

Chemical Analysis

The only demonstrable constituent in the purified R-body suspension was protein (85.7% from dry weight; 2.1 mg protein in 2.5 mg dry weight). With the anthrone method, we detected 0.13% glucose equivalent, but this value could have been measured due to sucrose contamination from the gradient centrifugation. Lipids were not detected. In R-body isolates not treated with DNase, we could not demonstrate ultraviolet absorption peaks typical for DNA.

Mitomycin C Induction

Addition of the antibiotic Mitomycin C to a log-phase culture, in many examples, induces prophages. In the case of defective lysogeny, the induction results in the production of "phage-like" particles (Lotz, 1976). In strain 2K1, treatment with Mitomycin C induced formation of bacteriophage tail-like particles (Fig. 8) without head, and unrolling of some R-bodies (Figs. 10 and 11). The tail-like particles in strain 2K1 were only present in cells induced with Mitomycin C. The tails are contractile. The extended particles measured 16 nm in diameter, with a total length of 60 nm, contracted particles exhibited a sheath diameter of 20 nm, a sheath length of 26 nm, a core diameter of 5 nm, a core length of 44 nm and a hexagonal basal plate with six tail fibres.

In one R-body suspension obtained from cells not treated with Mitomycin C we observed vesicles resembling anomalous phage heads (Fig. 9).

Unrolling of R-bodies within the cell was very rare.

However, after Mitomycin C treatment lysed cells with unrolled or partially unrolled R-bodies were relatively frequent (1–2%) (Figs. 10 and 11). In that case it was obvious that the R-bodies unrolled from the inner side (acute extreme) before cell lysis, or simultaneously with the lysis.

In no case could we observe formation of plaques in confluent cultures of strain 2K1 in Petri dishes.

Discussion

The R-bodies of the kappa particles were classified in the following two groups: 51 type R-bodies (unrolling from the inside; unrolling and rerolling depending on pH; both ends acute; without sheath; with helical phage like structures) and 7 type R bodies (unrolling from the outside; irreversible; not pH dependent; outer end blunt or irregular, inner end acute; with sheath; defective phages stick to the unrolled ribbons) (Preer et al., 1974). The 2K1 type R-bodies have some features in common with those from the 51 and from the 7 type, but differ in other aspects. They unroll from the inside in an irreversible manner and not depending on pH; the inner end is acute and the outer end blunt; without sheath; phage tail like particles were not observed sticking to the ribbons; they have an inner rhombical striation; the chemical composition is probably protein, as already described by Preer et al. (1966) for kappa R-bodies; they are smaller than the typical kappa R-bodies (about 0.5 μm in diameter). The 570-R-bodies are the most similar ones (approximately 0.2 μm in width and 5 μm in length); they are from the 7-type, with no sheath, and with spherical phage-like structures (Preer et al., 1972). Thus, strain 2K1 presents a new type of R-bodies, and it is the first free-living bacterium described with such an inner structure.

The number of 2K1 cells with detectable R-bodies increased in the stationary growth phase, probably because the cells had not such a high density as in the exponential growth phase, and the inner structures were then better visible in negatively stained preparations. However, it is also possible that the R-bodies, being coded by a defective prophage, whose induction causes death of the cell, but not its lysis occur more

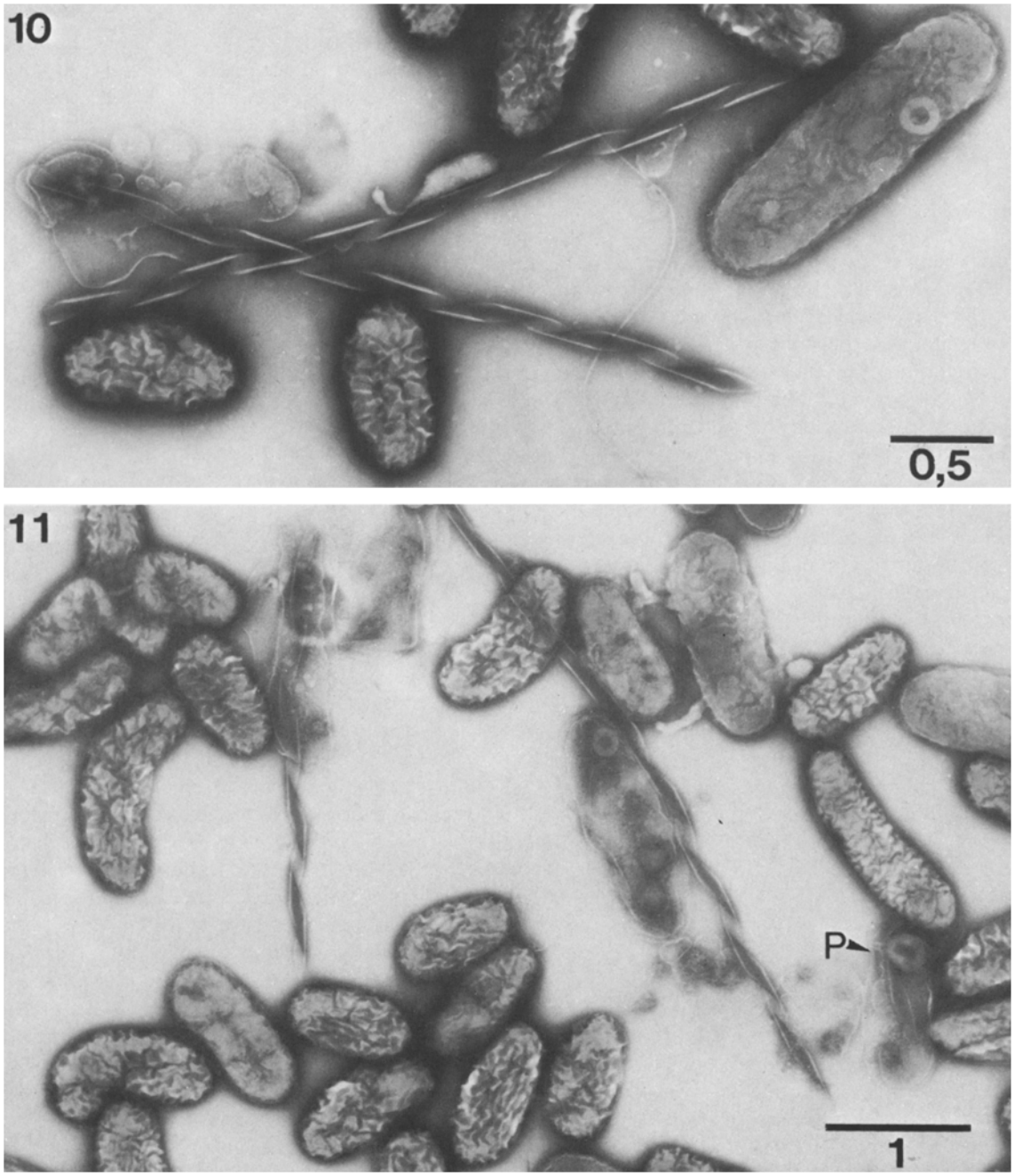

Fig. 10. R-body rolled within a cell, and two unrolled R-bodies. Cells treated with Mitomycin C

Fig. 11. Partially (*P*) and completely unrolled R-bodies in cells treated with Mitomycin C

frequently in stationary growth phase, i.e. at a moment when induction shows maximum effect. In this case the R-bodies are the result of a lethal synthesis (Preer et al., 1974). This possibility suggests an analogy with the particulate bacteriocins, particularly with the pyocine from *Pseudomonas aeruginosa* (Lotz, 1976). The dimensions and fine structure of the contractile tail like particles described by us are similar to pyocine R.

Results of Mitomycin C treatment, ultraviolet irradiation, and the presence of tail like particles in strain 2K1 are additional features in favour of Preer's hypothesis.

The kappa particles from the *Paramecium aurelia* species complex are described as endosymbionts which either contain R-bodies, or can produce endosymbionts with R-bodies, or were descendants from endosym-

bionts with R-bodies (Preer et al., 1974). Quackenbush (1977, 1978) proved recently by DNA homology studies that kappa can no longer be considered a closely related group of bacteria representing different strains of the same species. According to his findings, the kappa particles belong to different species of the genus *Caedobacter*, so that the presence of R-bodies is a distinctive characteristic of the genus. However, in the present paper, evidence has been presented that R-bodies are also present at least in one free-living bacterium, and it is the presence of the R-bodies which is the only relation that we can find between kappa and strain 2K1 (Lalucat et al., in preparation). It seems that the formation of R-bodies may be determined by a group of bacteriophages or extrachromosomal elements with convergent evolution, or with the same origin, occurring in quite different groups of bacteria.

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