Isolation of a New Free-living Bacterium Containing R-bodies

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(Received 14 May 1986)

Strain EPS-5028 is a free-living bacterium which contains R-bodies. By its morphology, biochemical characteristics and G + C content, strain EPS-5028 differs from the other known free-living R-body-containing bacteria, *Pseudomonas avenae* and *Pseudomonas taeniospiralis*. Phage and particles resembling phage heads were observed in cells induced with UV irradiation.

INTRODUCTION

R-bodies are refractile inclusions that are unique in nature. In its compact form an R-body is a long ribbon of protein wound into a tight roll, but it can also unroll into a long twisted filament. It is of taxonomic importance because it is the morphological characteristic which permits the genus *Caedibacter* to be distinguished from other bacterial endosymbionts of ciliates (Preer & Preer, 1984). Lalucat *et al.* (1978) were the first to describe R-bodies in a free-living bacterium: a new species of hydrogen-oxidizing bacterium, *Pseudomonas taeniospiralis*. R-bodies were subsequently reported in *Pseudomonas avenae* (Wells & Horne, 1983). R-bodies are presumably coded by a defective prophage, whose spontaneous induction determines the production of defective phages and R-bodies (Dilts, 1977). This group of bacteriophages, occurring in different groups of bacteria (Lalucat *et al.*, 1979), may have a common origin or may have arisen by convergent evolution. This report describes the isolation of an R-body-containing, nitrogen-scavenging bacterium which is different from *P. avenae* and *P. taeniospiralis*.

METHODS

Organism and growth. After screening extracellular polysaccharide-producing micro-organisms, bacterial strain EPS-5028 was isolated from a soil sample collected in Barcelona (Spain). Appropriate dilutions of the sample were inoculated on plates of tryptone soya agar (Oxoid) (TSA) and incubated at 30 °C. After 72 h incubation, colonies of mucoid appearance were picked, streaked onto fresh TSA plates and incubated for 72 h at 30 °C. Viscous-looking colonies were restreaked onto TSA slants and stored at 4 °C. Strain EPS-5028 was the most mucoid and was selected for our preliminary experiments (Congregado *et al.*, 1985).

Pseudomonas taeniospiralis DSM 2082 was used as a reference strain from nutritional characterization and DNA studies. *Azospirillum brasilense* ATCC 29145 and *Azotobacter vinelandii* ATCC 9140 were used as reference strains for nitrogen-fixation studies.

The modified mineral salts medium of Patt *et al.* (1974) was used for most experiments. The autoclaved basal salts medium was supplemented with filter-sterilized glucose to a final concentration of 0.5%. The pH was adjusted to 7 with 1 M-NaOH as required. Difco Noble agar (1%, w/v) was used to solidify the medium as necessary. Flasks containing 100 ml medium were inoculated with 0.1 ml of a cell suspension which had been washed three times with sterile phosphate buffer (pH 7) and adjusted to an OD₅₂₀ of about 0.2, and incubated aerobically at 30 °C for 72 h with shaking. Growth was measured as OD₅₂₀. For growth studies, cells were cultivated in a 10-l Braun Biostat S fermenter operating at 25 °C (impeller speed, 500 r.p.m.; pH 7; 80-100% O₂ saturation) containing 7 l mineral salts medium (with or without a source of combined nitrogen).

The ability of the isolate to grow in the absence of combined nitrogen was tested with a liquid nitrogen-free medium (Becking, 1981). Nitrogen fixation was estimated by the acetylene-reduction technique (Postgate, 1972), by measuring the ethylene production of cultures (5 ml) in 18 ml vials at 30 °C in both N-free liquid and semisolid medium. A loopful of bacteria from a previous 48 h culture was used as inoculum for each vial. After 24, 48 and 72 h, vials were closed with serum stoppers, and 0.2 ml C₂H₂ was injected into each vial. The amount of ethylene produced was determined after 1, 24 and 72 h by gas chromatography with a Perkin-Elmer model F-33 gas chromatograph equipped with a flame-ionization detector and operated at a column temperature of 50 °C. The carrier gas flow rate was 25 ml N₂ min⁻¹ in a column (0.32 × 50 cm) packed with 80–100 mesh Porapak N. Vials inoculated with *Azotobacter vinelandii*, *Azospirillum brasilense*, and heat-killed cultures were used as controls. Six replicate cultures were tested for each strain.

The media and conditions used for autotrophic growth were as given by Lalucat *et al.* (1979). The cells were cultivated autotrophically at 30 °C in 1-l Erlenmeyer flasks containing 100 ml mineral medium incubated in desiccators under an atmosphere of O₂/CO₂/H₂ (1:1:8, by vol.) with stirring. *P. taeniospiralis* was used as control. Three replicate cultures were tested for each strain.

Characterization. Phenotypic characterization was performed according to Cowan (1974). The G + C content of DNA was measured by the thermal denaturation method (Mandel & Marmur, 1968).

Electron microscopy. For negative staining a drop of the sample was applied to a copper grid coated with Formvar and carbon and stained with 2% (v/v) phosphotungstic acid (pH 7) or 4% (w/v) uranyl acetate (pH 4.8). Unstained samples were also examined. Drops of broth culture were placed onto carbon-stabilized Formvar films on 300 mesh copper grids and the specimens shadowed with platinum. For thin sections, preparations were fixed in 3% (w/v) glutaraldehyde in 0.1 M-sodium phosphate buffer (pH 7.2), treated with 1% (w/v) osmium tetroxide, dehydrated and embedded in Spurr resin, sectioned on a LKB Ultratome UM III ultramicrotome and double stained with uranyl acetate and lead citrate. The grids were examined in a Philips EM 301 electron microscope at an operating voltage of 80 kV.

Mitomycin C treatment and UV irradiation. Mitomycin C (0.1 mg ml⁻¹) was added to 10 ml cultures growing exponentially in mineral medium supplemented with sodium gluconate (0.25%) according to Lalucat *et al.* (1979). Cultures were irradiated for 90 s at a distance of 40 cm from a 15 W germicidal Philips lamp providing 253 nm UV light (Preer *et al.*, 1974). The irradiated cells were then incubated under the conditions described above.

Preparation of R-bodies. R-bodies for electron microscopy were prepared as described by Wells & Horne (1983). The resulting pellet was resuspended in 50 mM-phosphate buffer (pH 7).

RESULTS AND DISCUSSION

Exponentially growing cells of strain EPS-5028 in liquid medium were rod-shaped (length 1.3–2 µm, width 0.8–1 µm; Fig. 1*a*). During the stationary phase coccoid forms occasionally appeared. The Gram reaction was negative and the cell wall structure indicated a typical Gram-negative organization. Two polar flagella were observed (Fig. 1*a*) and cells were motile during the early phase of growth. Small pale colonies surrounded by abundant slime were formed on solid medium.

Transmission electron microscopy of cultures in several growth phases revealed R-bodies, normally one per cell (Fig. 1*b–d*). Phages or phage-like particles were not observed; they were found after UV treatment (Fig. 1*e*) but not after incubation with mitomycin C.

The production of one R-body has been shown to be determined by DNA sequences on a plasmid (Quackenbush & Burbach, 1983) and perhaps by phage or chromosomal genes in other bacteria (Quackenbush, 1978). Close genetic relationships between bacteria do not seem to be necessary for production of R-bodies. This is not altogether surprising, given that these structures are determined by nucleotide sequences in plasmids or phages or a small percentage of the nuclear DNA (Gibson, 1984). This had led to the suggestion that R-body formation occurs in different groups of bacteria. The presence of R-bodies in EPS-5028 supports this hypothesis because our strain is quite different from *Pseudomonas avenae* and *P. taeniospiralis*.

Table 1 lists the characters which differentiate EPS-5028 from other R-body-containing bacteria. The phenotypic and genetic characters of *P. avenae* were gathered from the literature. The DNA G + C contents of *P. avenae* (70.2 mol%) and *P. taeniospiralis* (60.4 mol%) are different from our isolate (65.1 mol%). There are several consistent differences between EPS-5028 and *P. avenae* and *P. taeniospiralis*, and on the basis of these distinct properties strain EPS-5028 cannot be assigned to either of these species.

Fig. 1. Electron micrographs of: (a) EPS-5028; (b) thin section of EPS-5028 containing an R-body; (c) an intact, coiled R-body; (d) an unrolled R-body; (e) phage from EPS-5028. Bar markers represent: (a) 0.5 μm , (b) 1 μm , (c) 0.1 μm , (d) 0.4 μm , (e) 50 nm.

In addition to differences in seven biochemical properties and G + C content, EPS-5028 may be distinguished from other R-body-containing bacteria by its apparent ability to grow in the absence of combined nitrogen. The growth rate with inorganic nitrogen and glucose (0.344 h^{-1}) was higher than that with glucose-containing, nitrogen-free medium (0.197 h^{-1}). Nitrogenase activity was not detected in liquid or semisolid nitrogen-deficient glucose medium. In this respect EPS-5028 resembles *Pseudomonas glathei* (Palleroni, 1984); but it can be distinguished from *P. glathei* in being positive for arginine dihydrolase, lactose and disaccharide utilization, and in its failure to grow on oxalate and on acid media.

Table 1. *Some differentiating characters of Pseudomonas taeniospiralis, Pseudomonas avenae and Pseudomonas sp. EPS-5028*

Test	<i>P. taeniospiralis</i>	<i>P. avenae</i> *	EPS-5028
Aerobic autotrophic growth with H ₂	+	ND	—
Heterotrophic denitrification	+	—	—
Oxidase	+	—	+
Urease	—	ND	+
Hydrolysis of gelatin	+	+	—
Hydrolysis of starch	—	+	—
Production of acids from carbohydrates	—	+	+
Poly- β -hydroxybutyric acid	+	+	—
Compounds serving as sole carbon source:			
Arabinose	+	—	+
Maltose	—	—	+
Sorbitose	—	+	+
Trehalose	+	—	+
Acetate	—	ND	+
Butyrate	—	ND	+
Citrate	—	ND	+
Isoleucine	+	ND	—

ND, No data available.

* Data from Palleroni (1984).

Strain EPS-5028 appears to be a new R-body-containing bacterium and it cannot be identified with any described *Pseudomonas* species. Its exact genus and species status is being investigated in our laboratory.

We thank Professor J. Lalucat for his comments and useful discussions during the course of this work and J. Blavia and R. Bargalló for their help with electron microscopy. This work was supported in part by grants from Direcció General d'Ensenyament Universitari of the Generalitat de Catalunya and the Comisión Asesora Científica y Técnica.

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