Characterization of Two "Metabacterium" sp. from the Gut of Rodents

2. Heteroxenic Cultivation and Proof of Dipicolinic Acid in "M. polyspora"*

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ABSTRACT. The vegetative cell of "Metabacterium polyspora" is "cucumber-shaped", about 21 x 5.7 μm, Gram-negative. Cylindrical endospores are best stained by Rakette and Ziehl–Neelsen staining. The bacterium reproduces by sporulation (2 to 8 endospores per cell) and by binary fission. Lateral, bow-like "hatching" of the endospores was seen. About 52 % of guinea pigs harbor 5 x 10⁶, 36 % below 2 x 10⁶ and 1 % more than 1 x 10⁸ "M. polyspora" in 1 g of caecal content. Dipicolinic acid was demonstrated using HPLC in the caecum homogenate from a guinea pig. The amount of it was proportional to the number of spores. Cultivation under strict anaerobic conditions did not succeed. It was possible to cultivate this giant endosymbiont in vitro in a heteroxenic culture incubated in a 5 % CO₂ atmosphere using liquid medium supplemented with cell-free filtrate of the caecum. The caecum filtrate containing undefined growth factor(s) is necessary for long-term culture. The replication rate was low. These findings suggest that the giant endosymbiont "M. polyspora" is a spore-forming prokaryote without the attributes of a strict anaerobe.

Until recently the only species known, representing the genus "Metabacterium" was "M. polyspora" occurring in the gut of the guinea pig and described by Chatton and Pérand (1913). The genus "Metabacterium" was classified in the last edition of Bergey's Manual under genera incertae sedis (Claus and Berkely 1986). It was shown that obviously several species differing morphologically occur in the gut of different herbivorous animals (Kunstý et al. 1988), also in the European hamster (Schiel et al. 1993). Morphological criteria strongly supported the prokaryotic character of "M. criceti", as the species from the gut of the European hamster (Kunstý et al. 1988; Schiel et al. 1993) was preliminary named. Uncertainty emerged as one symbiotic microorganism from the gut of the surgeonfish (Fishelson et al. 1985), very similar to "Metabacterium", was later classified as an eukaryote under the name Epulopiscium fishelsoni (Montgomery and Pollak 1988). It became evident that additional studies supporting the prokaryotic character of "Metabacterium" sp. were necessary.

As further studies of "M. criceti" were rendered impossible due to the abolition of the whole colony of these laboratory rodents (because of economic considerations), we concentrated our interest on "M. polyspora" living in the gut of guinea pigs.

MATERIAL AND METHODS

Cell morphology and prevalence. Light, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used as described by Schiel et al. (1993). Both native (phase contrast optics) and fixed and stained cells (Gram, Ziehl–Neelsen, Rakette, Lembach and Sons) were examined. The number of items in the stomach and the caecum of 300 conventional guinea pigs was evaluated by a semiquantitative counting method (Schagemann et al. 1988).

Identification of dipicolinic acid. The content of the whole caecum of a guinea pig selected for high numbers of "M. polyspora" was diluted with saline, homogenized, filtered (paper filter 589, Schleicher & Schüll) and centrifuged for 15 min at 1500 g. The pellet was resuspended in Ficoll, washed and centrifuged 3 times in saline and the number of "Metabacterium" cells counted microscopically. About

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1.5 mL of this caecal filtrate was centrifuged at 15 000 g for 5 min. The pellet was resuspended in 200 μL 0.2 mol/L potassium phosphate (pH 1.8) and incubated at 95 °C for 30 min. The supernatant was analyzed by reverse-phase HPLC on a 4.6 × 250 mm LiChrosorb RP8 column (Merck) at a flow rate of 1 mL/min using a 15-min gradient from 1 to 2.5% acetonitrile in 0.2 mol/L potassium phosphate (pH 1.8) (slightly modified according to Warth 1979). This procedure gave good separation from other extracted substances, most of which did not bind to the reverse-phase material.

In vitro cultivation. (1) Microflow Anaerobic System (type 22290, M.D.H., England, agency Nunc, Wiesbaden); atmosphere 82% N2, 10% CO2 and 8% H2; agar plates: Schaedler-anaerobicagar (Oxoid) and yeast–cysteine–blood agar; liquid media: Rosenow broth (Institut Pasteur, Marnes-la-Coquette), Wensink broth (Oxoid). (2) Gas-pak anaerobic jar (Oxoid) + Selectron filter AE 99/1 (Schleicher & Schüll, Dassel) + Columbia agar with blood (Oxoid) without or with gentamicin; yeast–cysteine–blood agar without or with gentamicin. (3) Tissue culture with stomach parietal cells of the guinea pig as monolayer (Sewing et al. 1983; Soll 1987) + Eagle's medium MEM, HEPES buffer, NaHCO3, 10% fetal calf serum + amphotericin and gentamicin (both Sigma); inoculum about 100 cells (in saline) harvested with micro-manipulator (Stachan and Kunst 1983; Schagemann et al. 1988). (4) Like (3) but enriched with Oscillospira guilliermondi, another giant intestinal bacterium. (5) Like (3) but inoculum consisting of pasteurized caecum content homogenate containing more "M. polyspora" cells supplemented with gentamicin and amphotericin. (6) Like (5) but the caecum filtrate originating from an animal with large numbers of "M. polyspora" was sterilized with γ-rays, dose 600–900 Gy (Gammatron 2, Siemens) and added (7.5%, V/V) with every change of the medium, i.e. every third day.

RESULTS

The endosymbiont studied is Gram-negative, 21 ± 4.39 μm long and 5.7 ± 1.1 μm wide, "cucumber"-shaped, slowly motile. Its wall has a structure characteristic of Gram-negative bacteria (not illustrated). The flagellar staining according to Lembach and Sons was positive but electron microscopy did not reveal flagella. It forms seldom 1, mainly 2–8 cylindrical endospores (Fig. 1) which stain well with Rakette and Ziel–Neelsen staining. In addition to reproduction through endospores, reproduction by binary fission could be seen (not illustrated). In 36% of animals (n = 300) the bacterium was not detected (below the limit of 2 million per gram content). In 52% of guinea pigs 5 × 10⁶ cells and in 1.2% more than 1 × 10⁸ cells per 1 g of caecal content could be counted. Constantly more microorganisms have been found in the caecum than in the stomach content.

Dipicolinic acid was demonstrated in the endospores (Fig. 2); the characteristic peak was proportional to their number (about 7.5 × 10⁷ and about 4.8 × 10⁸ — not illustrated).

All attempts to cultivate the bacterium in pure culture, whether under aerobic, semi-anaerobic or strictly anaerobic conditions, have been unsuccessful. Some low rates of replication could be seen in a heteroxenic in vitro culture by using Eagle's tissue culture medium + fetal calf serum + caecal filtrate + antibiotics on incubation in 5% CO₂.

Fig. 1. Two "Meta bacterium polyspora" cells containing 3 and 4 endospores, respectively; SEM.