Phylogenetic status of *Anaerobacter polyendosporus*, an anaerobic, polysporogenic bacterium

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The almost complete sequence of the 16S rRNA gene of the Gram-positive polysporogenic bacterium *Anaerobacter polyendosporus* was determined. This allowed phylogenetic analysis of *A. polyendosporus* by comparing sequences of the 16S rRNA gene of this bacterium to similar genes of other Gram-positive bacteria. It was shown that this polysporogenic bacterium belongs to the *Clostridium* cluster I, subcluster A. Phylogenetically, *A. polyendosporus* is distantly related to another polysporogenic, but non-cultivatable, bacterium, *'Metabacterium polyspora' and can be satisfactorily clustered within the saccharolytic clostridia with a low DNA G+C content grouped in subcluster A. *A. polyendosporus* was most closely related to *Clostridium intestinale* (94.8 % identity of 16S rRNA genes) and *Clostridium fallax* (93.1 %). Like other members of the *Clostridium* cluster I, subcluster A, *A. polyendosporus* possesses such common phenotypic features as a Gram-positive cell wall structure, anaerobiosis, derivation of energy from carbohydrate fermentation yielding butyric acid among other organic acids and the capacity for endogenous spore-formation. However, the scale of evolutionary change in the 16S rRNA gene between *A. polyendosporus* and phylogenetically related *Clostridium* species does not correspond to the profound changes in the phenotype of *A. polyendosporus*. Distinctive phenotypic features of the latter are large cell size, polysporogenesis (up to seven spores per cell), alternative modes of development and an unusual membrane ultrastructure.

**Keywords:** *Anaerobacter polyendosporus*, 16S rRNA gene, *Clostridium*, ultrastructure, endospore, polysporogenic bacteria

At present, two genera of bacteria capable of forming more than two endospores in one cell are known. These are *Metabacterium* (forming up to nine spores) (Krassilnikov, 1949) and *Anaerobacter* (up to five spores) (Duda et al., 1987). Unlike the non-cultivated *Metabacterium*, a representative of the genus *Anaerobacter* was obtained in pure culture and can thus be used as a model organism in studies of polysporogenesis in bacteria. The results of these investigations are important for the estimation of the potential capabilities of the prokaryote cell, the elucidation of cellular and molecular mechanisms of sporogenesis and also for investigations on the regulation of cell differentiation and evolution of bacteria.

Data have recently been obtained on the almost complete sequence of the *'Metabacterium polyspora' 16S rRNA* gene, making it possible to define its phylogenetic relationship to other micro-organisms (Angert et al., 1996; Pace, 1996). However, the phylogenetic position of *Anaerobacter* is still obscure. The present work is focussed on the determination of the sequence of the 16S rRNA gene of *Anaerobacter polyendosporus* and on obtaining information on the ultrastructural organization of vegetative cells and spores, to clear up the question of the phylogenetic status of this bacterium.

The bacterium *A. polyendosporus* (strain PS-1†), the

† Deceased March 30 1998.

The GenBank accession number for the 16S rDNA sequence of *Anaerobacter polyendosporus* strain PS-1† is IG222546.
properties of which have been described previously (Duda et al., 1987), was used in our investigation. The strain was cultivated on potato agar, to which 0·5 % glucose (or galactose), 0·1 % yeast extract and 0·04 % sodium thioglycollate were added, and on synthetic solid and liquid media composed of the following compounds per litre of distilled water: KH₂PO₄, 0·33 g; NaHCO₃, 1·5 g [mineral base of Pfennig medium (Pfennig, 1965)]; trace element solution (Pfennig & Lippert, 1966), 1 ml; agar (when necessary), 20 g; galactose or glucose, 0·3–5·0 %. The medium was autoclaved in an atmosphere of CO₂/H₂/N₂ (10·5:85). The bacteria were incubated in anaerobic jars (Oxoid) in an atmosphere with a final CO₂ concentration of 8–10 %. Cultivation was performed at 28 °C.

16S rDNA of *A. polyendosporus* was isolated and purified by the procedure of Marmur (1961) and then amplified by Vent, thermostable DNA polymerase (NEB) in a mixture containing: 1 × Thermopol buffer (NEB); 0·2 μg *A. polyendosporus* chromosomal DNA; 20 μM oligonucleotide primers 5' A and pH' (Edwards et al., 1989); 2·5 mM dNTP; 2·5 mM MgCl₂ and 1 U Vent. After denaturation for 3 min at 94 °C, the reaction mixture was taken through 30 rounds of amplification (54 °C, 1 min; 72 °C, 1·5 min; 94 °C, 1 min). Amplified products were purified by electrophoresis in 0·8 % agarose. The DNA band of interest was cut from the gel and eluted by centrifugation through siliconized glass fibre.

We sequenced 1500 nucleotides on both strands by the method of Sanger et al. (1977) with Sequenase 2·0 (USB). All procedures were carried out in accordance with the supplier’s protocols.

Cells of *A. polyendosporus* were examined by light, phase-contrast and electron microscopic techniques (JEM-100C and JEM-100B; JEOL). To obtain data on cell ultrastructure, electron microscopy of thin sections and replicas of freeze-fracture was carried out. To obtain thin sections, samples were fixed by the method of Ryter et al. (1958), embedded in Araldite-Epon according to conventional procedures, thin-sectioned with an LKB Ultrotome and then stained with lead citrate according to Reynolds (1963). To obtain freeze-fracture of cells, we used a special device for super-fast freezing of the cell suspension as a thin film (thickness of ~ 2 mm), placing it between two copper electron-microscopic grids (Fikhte et al., 1973). Samples were frozen in liquid propane that had first been cooled to −196 °C with liquid nitrogen. Cells were fractured and etched (1 min) in a JEE-4x vacuum evaporator at 0·3 mPa and a sample temperature of −100 °C. The fracture faces were shadowed with a platinum/carbon mixture and coated with carbon.

The 16S rDNA gene sequences of *A. polyendosporus* and other closely related micro-organisms (Table 1)
Fig. 1. Phylogenetic position of *A. polyendosporus* among closely related members of the *Clostridium* group. The 16S rDNA sequence of *Bacillus cereus* was used as an outgroup. The unrooted phylogenetic tree was derived from 16S rDNA sequences and created by using the neighbour-joining method and $K_{\text{nucl}}$ values. The numbers on the tree indicate bootstrap values (percentage) for the branch points. The strains used and the nucleotide sequence accession numbers are indicated in Table 1.

Among subcluster A of the clostridia (Fig. 1), there are species with strong saccharolytic activity, forming as fermentation products acetic and butyric acids, ethanol, butanol, $H_2$, and $CO_2$. This is also the case for *A. polyendosporus*.

Unlike phylogenetically related clostridia, *A. polyendosporus* can form several endospores in one cell. On synthetic medium with galactose (0.1–0.3% w/v), some cells may produce up to seven endospores (Fig. 2). Another peculiarity of the development cycle of the bacterium is the formation of polygonal cells (Fig. 3) in growth medium with an abundant quantity of carbohydrate (potato agar plus 0.5–1.0% w/v glucose or galactose). Under these conditions, sporulation in large, spherical cells is completely suppressed. However, finer, sometimes flat, polygonal cells, formed at a late stage of cultivation, are capable of sporulating (V. I. Duda, N. E. Suzina & V. V. Dmitriev, unpublished results).
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One more unique cytological peculiarity of *A. polyendosporus* is the formation of extensive lipid leaves (pieces) in the cytoplasmic membrane, located between the outer and internal lipid layers of the membrane (Fig. 4). These leaves can also be designated as intramembrane structures or inverted membranes (V. I. Duda & N. E. Suzina, unpublished results). Similar structures were not observed in other spore-forming anaerobes or aerobes (Duda, 1982; Vaisman, 1981). Intracytoplasmic (mesosome-like) structures in *A. polyendosporus* cells are rare and take the form of plates that are localized in the cytoplasmic periphery near the cytoplasmic membrane.

The endospores have the distinctive ultrastructure of endospores of representatives of the genus *Clostridium*: they possess spore coats, exosporium, inner and outer membranes, cortex and core. These structures can be observed easily either in ultra-thin sections or in replicas of freeze-fractured spores (Figs 5, 6).

The analysis of the 16S rRNA gene sequence has shown that *A. polyendosporus* is phylogenetically close to the cluster of saccharolytic clostridia with low DNA G+C content (*C. intestinale, Clostridium butyricum, Clostridium saccharoperbutylicenon* and other related species). The general properties of bacteria within the cluster are: (i) Gram-positive type cell wall; (ii) carbohydrates fermented with formation of organic acids (acetic, butyric, propionic) and ethanol, butanol, H₂, and CO₂; and (iii) endogenous spore-formation. *C. intestinale* appeared to be the closest relative of *A. polyendosporus* (94.8% identity); *Clostridium pasteurianum* is more remote (90.2% identity). Among the related clostridia, two species, ‘*Clostridium corinoforum*’ and ‘*Clostridium favososporum*’ have previously been described by Duda & Makaryeva (1977) and Krassilnikov et al. (1971a, b).

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**Fig. 4.** Electron micrograph of a freeze-fracture of cytoplasmic membranes of a vegetative cell. PF CM, Protoplasmic face of cytoplasmic membrane; CW, cell wall; IL, intramembrane lipid layers, deficient in intramembrane particles. The direction of shadowing is indicated by an arrowhead.

**Fig. 5.** Electron micrograph of ultra-thin section of a sporulating cell. CW, Sporangium cell wall; E, exosporial layers; Ct, spore coats; CM, cytoplasmic membrane; OM, outer spore membrane; IM, inner spore membrane; C, cortex; PW, primordial cell wall; Co, core.

**Fig. 6.** Electron micrograph of a freeze-fracture of a sporulating cell. CW, Sporangium cell wall; E, exosporial layers; Ct, spore coats; OM, outer spore membrane; IM, inner spore membrane. The direction of shadowing is indicated by an arrowhead.
The data obtained are consistent with the results of 16S rRNA sequence analysis of this polysporogenous bacterium, showing that *A. polyendosporus* is a remote but specific relative of the phylogenetic branch of clostridia that includes saccharolytic bacteria such as *C. butyricum* and *C. pasteurianum* (Chumakov, 1987; Duda *et al*., 1987). However, the scale of the evolutionary changes in the *A. polyendosporus* 16S rDNA gene, in comparison with those of related *Clostridium* species, does not correspond to the large changes in the phenotype of this polysporogenous bacterium. Among the peculiar features of this bacterium, the following could be mentioned: (i) large cell size (up to 4–6 μm in spherical forms); (ii) the ability to form up to seven endospores per cell (only five were observed previously; Duda *et al*., 1985, 1987); (iii) the alternate pathway of development in the life cycle, i.e. some cells in medium with a high carbohydrate concentration were of polygonal form capable of sporulation; (iv) peculiarity of the cytoplasmic membrane ultrastructure (formation of intramembrane structures as lipid leaves).

Essentially, the situation is the same when comparing data on molecular systematics and phenotypic characteristics of the other polysporogenous (but non-cultivable) bacterium *M. polyspora* and its nearest relative, the polysporogenous anaerobe *Clostridium lentocellum* (Angert *et al*., 1996; Pace, 1996). The data obtained show that *A. polyendosporus* is not a close relative of *M. polyspora* (79.9% identity). There are also the significant differences in the phenotypic characteristics of *A. polyendosporus* and *M. polyspora*. Moreover, the cells of *M. polyspora* are Gram-negative, due to their cell wall structure (Kunstfär et al., 1988), whereas *A. polyendosporus* is a Gram-positive bacterium (Duda *et al*., 1987). The lengthened, cylindrical form of *M. polyspora* endospores differs sharply from the egg-like and spherical endospores of *A. polyendosporus*. In addition, the ecological niches of these two bacteria are considerably different: *M. polyspora* is found in the guts of some animals whereas *A. polyendosporus* is a soil saccharolytic bacterium.

Thus, the description of *A. polyendosporus* has been supplemented with the observation of some cytological properties and several taxonomic attributes, together with the sequence of the 16S rRNA gene which has allowed the phylogenetic position of this bacterium to be determined. The type strain (PS-1<sup>T</sup> = VKM B1724<sup>T</sup>) of the species *A. polyendosporus* is kept in the All-Russia Collection of Microorganisms.

**Acknowledgements**

The authors are grateful to Professor L. V. Kalakoutskii for his attention to the present work. The authors also thank Ms Lily P. Chigaleychik for her help in cultivation of anaerobic bacteria and Mr N. I. Basovsky for his help with electron-microscopic studies.

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