NOTE

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^T), the

† Deceased March 30 1998.

The GenBank accession number for the 16S rDNA sequence of *Anaerobacter polyendosporus* strain PS-1^T is IG222546.

Table 1. Selected members of the genus *Clostridium* and closely related micro-organisms used for data analysis

Organism	16S rRNA GenBank no.	Strain
Clostridium absonum	X77842	DSM 599^T
Clostridium butyricum	X68176	ATCC 43755
'*Clostridium corinoforum*'	X76742	DSM 5906
Clostridium fallax	M59088	ATCC 19400^T
'*Clostridium favososporum*'	X76749	DSM 5907
Clostridium intestinale	X76740	DSM 6191^T
Clostridium malenominatum	M59099	ATCC 25776^T
Clostridium novyi	M59100	ATCC 17861^T
Clostridium baratii	M59102	VPI 1586
Clostridium paraputrificum	X73455	DSM 2630^T
Clostridium pasteurianum	M23930	ATCC 6013^T
Clostridium puniceum	X71857	DSM 2619^T
Clostridium quinii	X76745	DSM 6736^T
'*Clostridium saccharoperbutylacetonicum*'	U16122	N1-4
Clostridium scatologenes	M59104	ATCC 25775^T
Clostridium sporogenes	X68189	ATCC 3584^T
Clostridium tetanomorphum	X68184	NCIMB 11547
Clostridium botulinum type A	X68185	NCTC 7272
Clostridium botulinum type B	X68186	NCTC 7273
Clostridium lentocellum	X76162	DSM 5427^T
Sarcina maxima	X76650	DSM 316^T
Sarcina ventriculi	X76649	DSM 286^T
'*Metabacterium polyspora*'	U22332	M 1.6

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: KH_2PO_4, 0·33 g; NH_4Cl, 0·33 g; $MgCl_2.2H_2O$, 0·33 g; $CaCl_2.6H_2O$, 0·33 g; $NaHCO_3$, 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 $CO_2/H_2/N_2$ (10:5:85). The bacteria were incubated in anaerobic jars (Oxoid) in an atmosphere with a final CO_2 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 $Vent_r$ thermostable DNA polymerase (NEB) in a mixture containing: 1 × ThermoPol buffer (NEB); 0·2 µg *A. polyendosporus* chromosomal DNA; 20 pM oligonucleotide primers *pA* and *pH′* (Edwards *et al*., 1989); 2·5 mM dNTP; 2·5 mM $MgCl_2$ and 1 U $Vent_r$. 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)

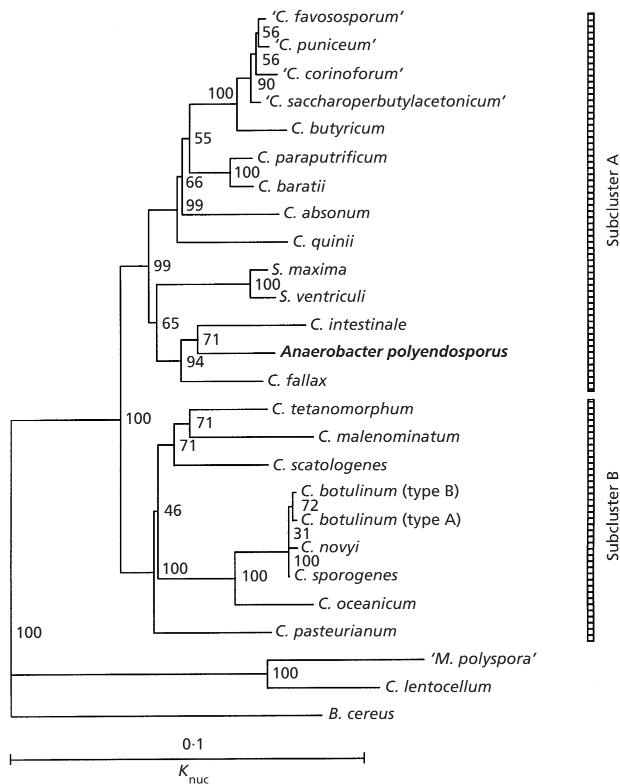

Fig. 1. Phylogenetic position of *A. polyendosporus* among closely related members of the *Clostridium* group. The 16S rDNA sequence of *Bacillus cereus* was used as an outgroup. The unrooted phylogenetic tree was derived from 16S rDNA sequences and created by using the neighbour-joining method and K_{nuc} 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.

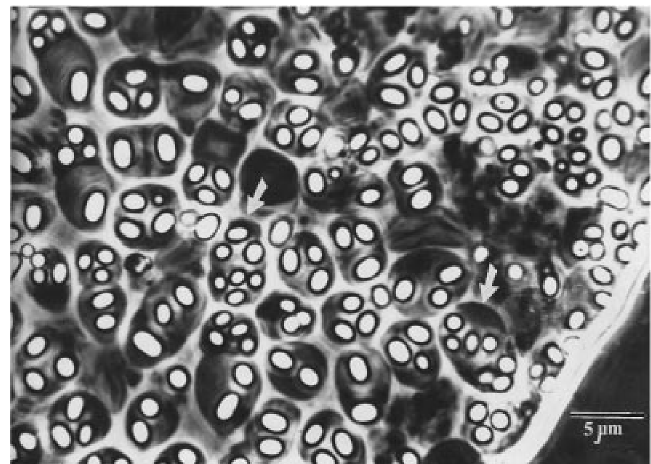

Fig. 2. Phase-contrast micrograph of sporulating cells. The arrow indicates a sporangium with seven endospores.

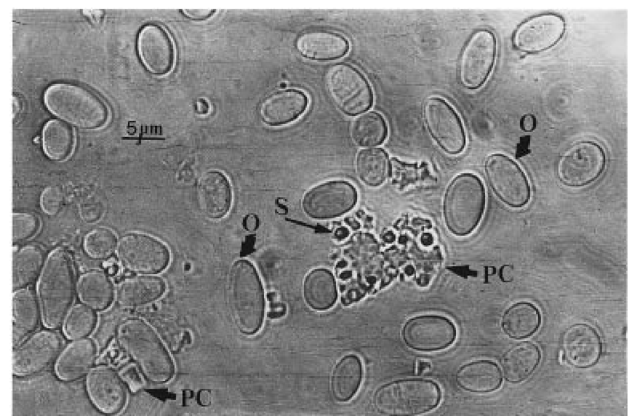

Fig. 3. Micrograph of native cells, obtained with an ordinary light microscope. O, Oval cells; PC, polygonal cells; S, spores.

were analysed. Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura & Ohta, 1972) and a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). The topology of trees was evaluated by bootstrap analysis of the sequence data with CLUSTAL W software (Thompson *et al.*, 1994). The 16S rDNA gene sequence of *Bacillus cereus* (strain NCTC 11143; GenBank accession no. X55063) was used as an outgroup.

Fig. 1 represents the phylogenetic tree constructed from the results of computer processing of data obtained and information on the sequences of 16S rDNA of closely related *Clostridium* species taken from the EMBL/GenBank/DDBJ databases. *A. polyendosporus* forms part of the cluster of species corresponding to cluster 1, subcluster A of Collins *et al.* (1994). *A. polyendosporus* appears to be phylogenetically close to clostridia with low DNA G+C content within subcluster A. *Clostridium intestinale* is the closest relative of *A. polyendosporus*, since their 16S rDNA sequences are 94·8 % identical.

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).

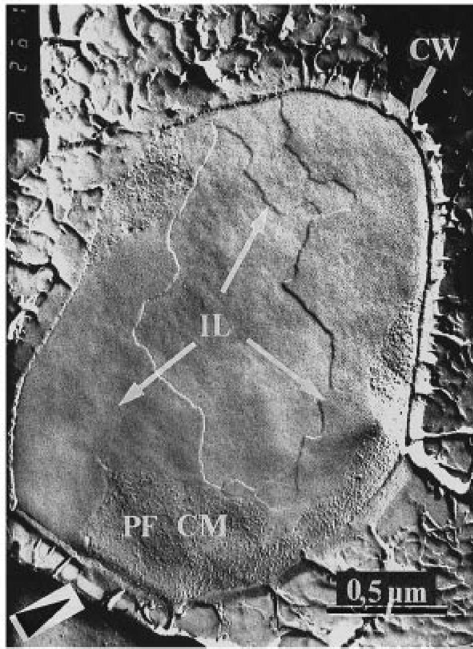

Fig. 4. Electron micrograph of a freeze-fracture of cytoplasmic membranes of a vegetative cell. PF CM, Protoplasmic face of cytoplasmic membrane; CW, cell wall; IL, intramembrane lipid layers, deficient in intramembrane particles. The direction of shadowing is indicated by an arrowhead.

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 saccharoperbutylacetonicum*' 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, H_2 and CO_2; 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).

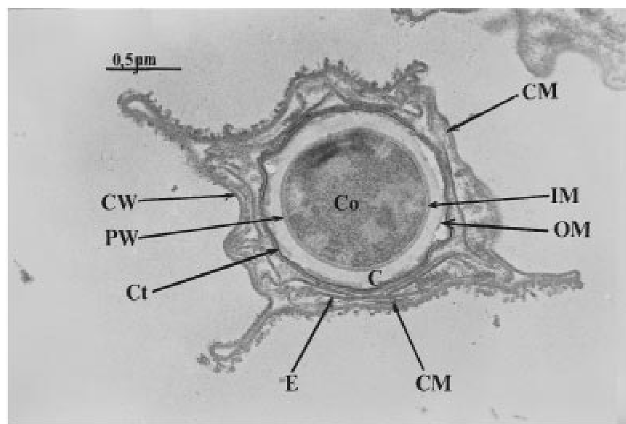

Fig. 5

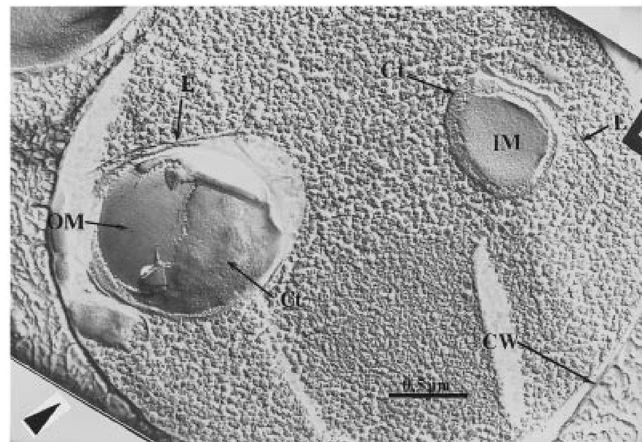

Fig. 6

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 5S rRNA sequence analysis of this polysporogenic 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 rRNA 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 polysporogenic (but noncultivatable) bacterium '*M. polyspora*' and its nearest relative, the polysporogenic 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 (Kunstýř *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^T = VKM $B1724^T$) of the species *A. polyendosporus* is kept in the All-Russia Collection of Microorganisms.

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