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ORIGINAL PAPER

Chemostat-induced uneven division of *Bacillus subtilis*

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Abstract Anomalous forms of *Bacillus subtilis* A32 produced by prolonged cultivation in a chemostat under nitrogen limitation are described. A change in the cultivation conditions brought about a transformation of these forms to bacillar rods. The transformation was gradual and lasted for several generations.

Keywords Chemostat · *Bacillus subtilis* · Defective division · Ultrathin sections

Introduction

One of the leading ideas connected with the foundation of the Institute of Microbiology was complex analysis of microbial life. An excellent overview of the research performed at the institute, where the dominating field of activity was continuous cultivation of microorganisms, was written by Štrbáňová in 2006 [1]. The ultrastructure of bacterial cells was studied in the Department of Electron Microscopy at a level comparable to other European laboratories at that time. During the study of α-amylase production in chemostat by strains of *Bacillus subtilis* A32 [2], the atypical morphology described below was observed.

To Vladimir Munk, with respect and affection.

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Materials and methods

Strain

Bacillus subtilis A32, *Bacillus amyloliquefaciens* 3129 DBM.

Cultivation

Cultivation was carried out in a 2.5-L fermentor (Development Workshop of the Czechoslovak Academy of Science), with working volume of 1 L at 1,000 rev/min and aeration rate of 1 volume air to 1 volume medium per min at temperature of 30°C.

Medium composition

The following medium compositions were used: (A) normal medium: 0.5% $(NH_4)_2HPO_4$, 0.5% Na citrate, 0.2% NaCl, 0.02% $MgSO_4$, 0.01% KCl; (B) limiting medium: 0.2% $(NH_4)_2HPO_4$, 0.15% K_2HPO_4, 0.5% Na citrate, 0.2% NaCl, 0.02% $MgSO_4$, 0.01% KCl. In both media, pH was adjusted to 7.2 and 2% glucose was the sole carbon source.

Cultivation conditions

Specific cultivation conditions for the formation of the spherical forms of *B. subtilis* were as follows: The spheres were formed only in continuous cultivation, in nitrogen-limited medium, after a steady state was reached and culture maintained at constant dry weight. The cultivation time required for the formation of the anomalous forms was directly proportional to the dilution rate D; at $D = 0.05$–0.1, spheres appeared after 3–5 days; at $D = 0.15$–0.20, they appeared within 7–10 days of cultivation [3].

Electron microscopy

Cells were fixed with 1% OsO_4 and embedded in Vestopal using the standard technique of Kellenberger et al. [4]. Agar blocks were saturated for 2 h with 0.5% uranyl acetate before dehydration. Saturation with Vestopal was done by gradually evaporating acetone from 25% solution of Vestopal. After polymerization, the blocks were sectioned with glass knives using a LKB 4800 A-Ultrotome. Grids were observed using a Philips EM 300 microscope [3].

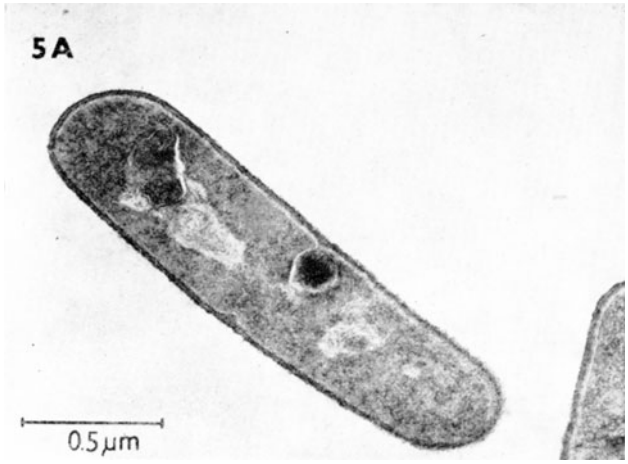

Fig. 1 Ultrathin section through the bacillary form of *Bacillus subtilis* after 12 h of continuous cultivation. *Scale line* 0.5 µm

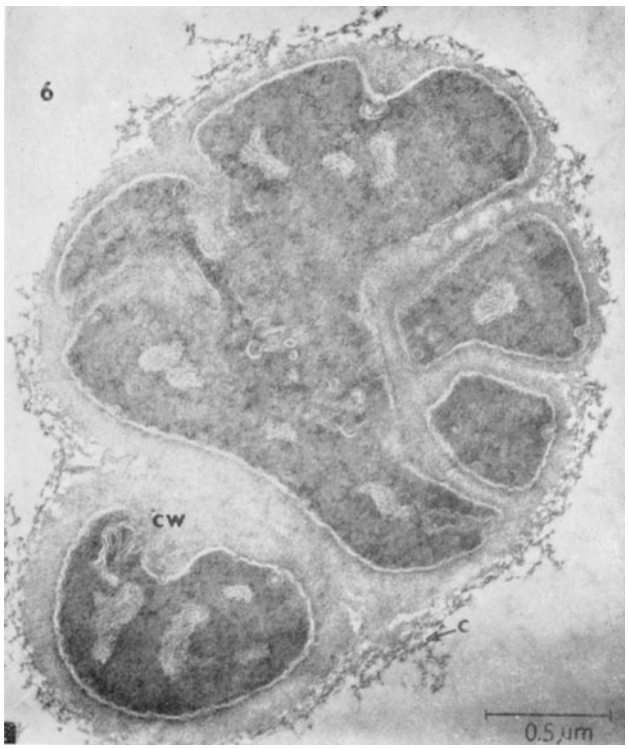

Fig. 2 Ultrathin section through altered forms of *Bacillus subtilis* after 90 h of continuous cultivation showing deformed ultrastructure. The septa are initiated irregularly; the cells do not separate. *cw* cell wall. *Scale line* 0.5 µm

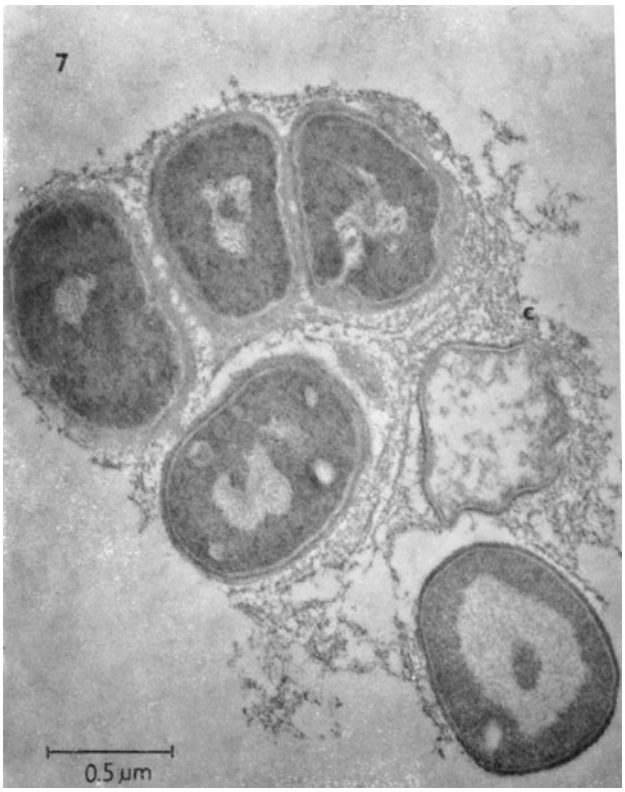

Fig. 3 Ultrathin section through altered forms of *Bacillus subtilis* after 90 h of continuous cultivation. Cell wall is thick; the capsule (*c*) forms concentric layers. *Scale line* 0.5 µm

Results and discussion

Bacillary rods (Fig. 1) were transformed into irregular spherical forms (Fig. 2). Jana Čáslavská [5], who analyzed these "freaks" using ultrathin sections and transmission electron microscopy [3], revealed deep changes of the cell wall (CW) (Figs. 3, 4). The spherical forms were actually clusters of cells with irregularly formed division septa, and cells formed in this way did not separate but remained joined. Deformity of the cells was transient, because when they were removed from the chemostat and cultivated in batch manner in rich complex medium, the abnormalities disappeared after a few cell cycles and cells returned to standard shape [6]. Cell suspension taken from the chemostat was supplemented by casamino acids and cultivated in a reciprocal shaker (Fig. 5). Samples were taken after 12 h; the recovery is shown in Figs. 6–8.

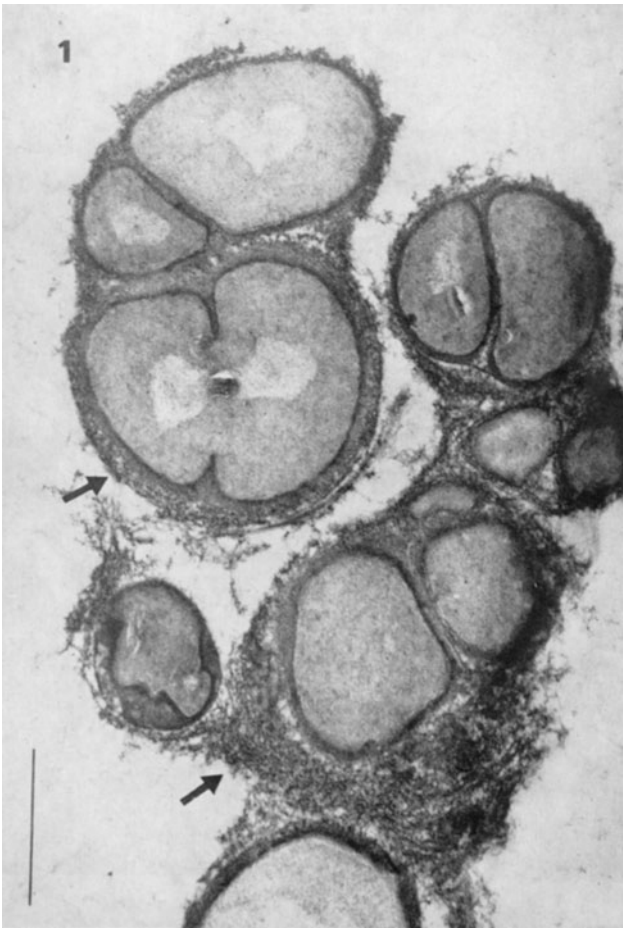

Fig. 4 Ultrathin section through altered forms of *Bacillus subtilis* after 90 h of continuous cultivation. Clusters of irregularly dividing cells are enfolded in a polysaccharide capsule (*arrow*). *Scale line* 1 μm

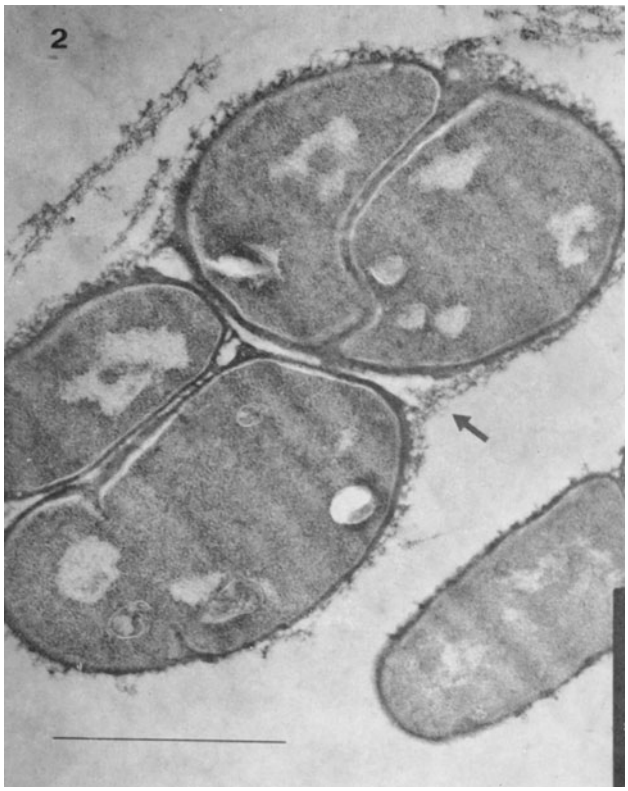

Fig. 5 *Bacillus subtilis* after a change of cultivation conditions—transfer from chemostat to a flask (reciprocal shaker) supplemented by casamino acids. Individual groups of anomalous cells are released. *Arrow* indicates the remnants of the capsule. *Scale line* 1 μm

The prevailing opinion in those days concerning the rules of division of rod-shaped bacteria was based on genetic background, postulated by Rogers et al. [7, 8], who studied conditional mutants of *B. subtilis* that grow as irregular cocci under restrictive conditions but as normal rods under permissive conditions. The CW is above all composed of peptidoglycan consisting of linear heteropolysaccharide backbone chains that are cross-linked, via short peptides, to form a net-like molecule. Most mutations identified as affecting cell shape [9, 10] were in genes encoding proteins involved in CW synthesis or formation. Based on our observations [2] we assumed that the cells lost the ability to form regular septa and to divide as a consequence of long-term N limitation.

Today it is known that, during cell division, FtsZ is the first protein to move to the division site [11] and is essential for recruiting other proteins that produce a new CW between the dividing cells. FtsZ's role in cell division is analogous to that of actin in eukaryotic cell division, but unlike the actin–myosin ring in eukaryotes, FtsZ has no known motor protein associated with it. The origin of the cytokinetic force thus remains unclear, but it is believed that localized synthesis of new CW produces at least part of this force. A major breakthrough came in 2001 when the highly conserved *mreB* family of genes bearing sequences of distant but true actin homologs were published [12, 13]. The scaffold theory of CW formation is supported by information that shows that the formation of the ring and localization to the membrane require the concerted action of a number of accessory proteins. ZipA or the actin homolog FtsA permit initial FtsZ localization to the membrane [11]. Following localization to the membrane, division proteins of the Fts family are recruited for ring assembly [12]. Many of these proteins, such as FtsW, FtsK, and FtsQ, are involved in stabilization of the Z ring and may also be active participants in the scission event [14]. The number of proteins found to be taking part in final shaping of Gram-positive bacteria has been increasing every year.

We were able to declare in 1972 [2] that, during continuous cultivation under N limitation, when the time

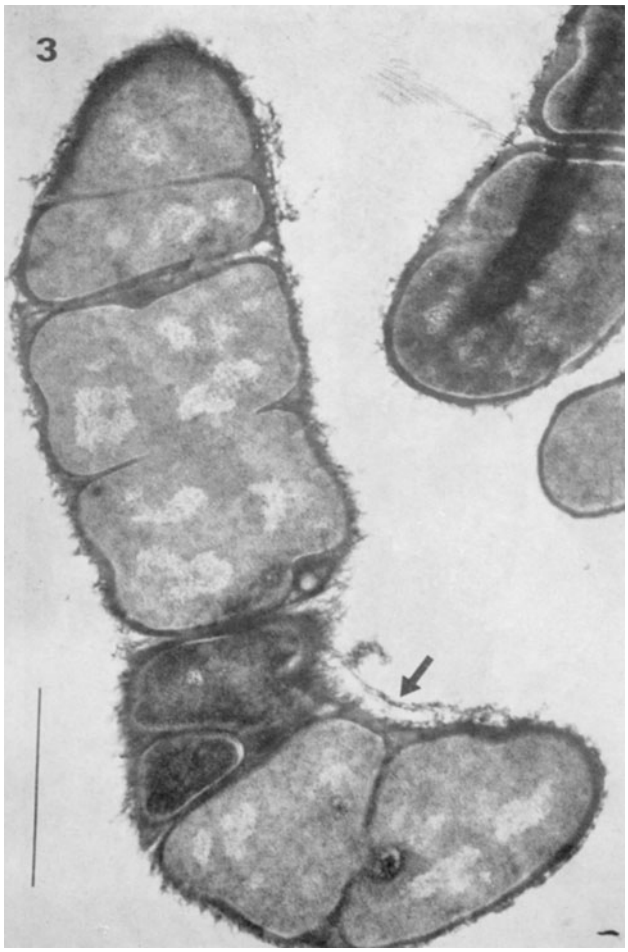

Fig. 6 Released cell groups are transformed to elongated filaments of anomalous cells. *Arrow* indicates the remnants of the capsule. *Scale line* 1 μm

factor is absent and cells can be grown for indefinite periods of time under precisely defined limiting nutrition conditions, a similar effect as for cultivation under restrictive temperature [7–9] is achieved. Our conclusion was that synthesis of the enzymes (proteins) playing a key role in cell division is limited under the above-stated conditions.

Since the main task of *B. subtilis* A32 cultivation in chemostat was to find optimal conditions for α-amylase production [2], the problem of undisciplined cell division was solved by addition of 2 g/L casamino acids to mineral medium, and the morphology of cells became regular.

What motivated these considerations? In this communication, evidence of deep, complex changes of the bacillar cell wall is provided. Until then, similar experiments using chemostat cultivation leading to changes in bacillar morphology had not been carried out and described, and their putative genetic or molecular nature had not been elucidated. In the 1970s, the author could not continue this line

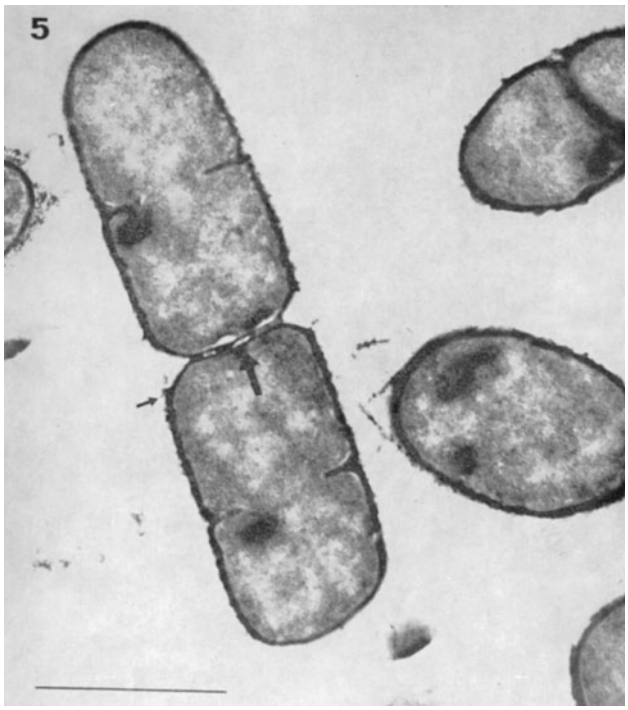

Fig. 7 New septa begin to form. Mesosomes are observable. Old wall peels off on the surface (*small arrows*). Separated cells disintegrate (*heavy arrow*). *Scale line* 1 μm

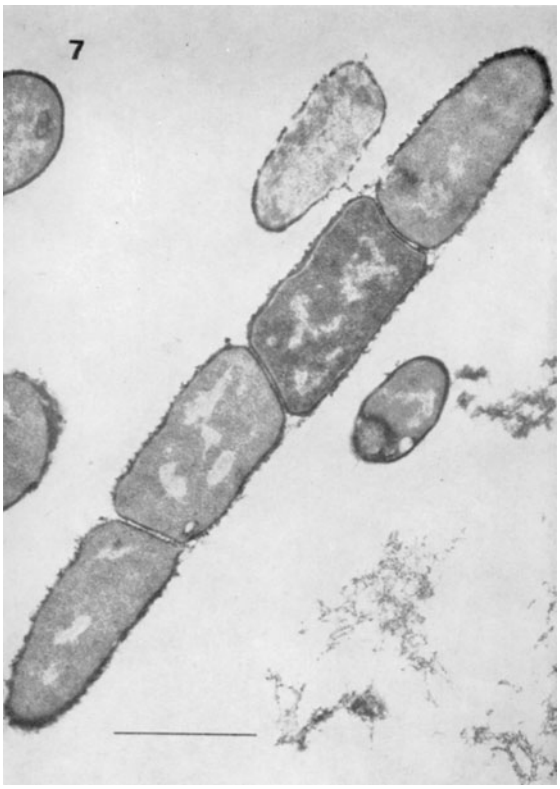

Fig. 8 Cells of *Bacillus subtilis* after 12 h of cultivation on a reciprocal shaker. Most cells have a regular bacillar shape. *Scale line* 1 μm

of exploration. After Ivan Málek's forced departure, the Institute of Microbiology was a different and strange place. The supervisor of the author, Vladimir Munk, emigrated to the USA in 1969. Despite these difficult circumstances, the results achieved exactly during the Prague Spring of 1968 were finally published in *Folia Microbiologica* but without the possibility to study this phenomenon in depth. There was no other choice but to believe that someone more prepared and well equipped would solve this enigma in the near future.

Epilogue

Strain *B. subtilis* A32 was later reclassified as *B. amyloliquefaciens* and is kept in the ICTP collection as 3129 DBM.

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