PLEOMORPHISM IN BACTERIAL CELLS

1. FILAMENT FORMATION IN LACTOBACILLUS DELBRUECKII AND ITS RELATION TO VITAMIN B₁₂

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As cell division is one of the most fundamental phenomena in life cycle, innumerable cytological and cytochemical studies have been achieved in both fields of botany and zoology. However, bacteriologists have been largely handicapped from these fields, because the cell size of bacteria is too small for application of general cytological methods. Therefore, almost no studies on the mechanism of bacterial cell division have hitherto appeared except those by workers dealing with the problem of filamentous cells in hoping to gain some information through consideration that abnormal elongation of cells must be resulted by the inhibition of cellular division without affecting cell growth.

Recently, WEBB (1) has observed that some Gram-positive rod bacteria become filamentous when cultured in a magnesium-deficient medium. Abnormal elongation was also observed by DANIEL et al. (2) with the cells of Lactobacillus leichmanii and further by CHAPLIN and LOCKHEAD (3) with the cells of Arthrobacter sp. cultured in vitamin B₁₂ deficient media.

Plemorphism at the cells of Lactobacillus delbrueckii has long been known since its first description by HENNEBERG in 1926. Actually, the cell length of this species is surprisingly variable in certain conditions.

We have made attempts to clarify the reason for the appearance of abnormal formed cells during the culture of L. delbrueckii. As the result of this study we have observed the following phenomenon: an extraordinarily large amount of B₁₂ is necessary for normal cellular division.

In this paper the cytological effects of B₁₂ on cell growth and cellular division in the culture of L. delbrueckii are presented.

METHODS

Organism: Lactobacillus delbrueckii No. 1, a type culture in this laboratory, authenticated as a thermophilic homofermenter forming D(−)-lactic acid, has been used throughout this investigation.

Culture Media: For the purpose of harvesting the inoculum cells of the
organism, glucose—yeast extract—peptone medium (GYP) was applied. For the determination of the biochemical action of B_{12}, Difco’s B_{12} assay medium was used as a basal medium.

**Culture:** Culture was carried out at 45° in test tubes. Inoculum cells were prepared as follows: after incubation for 1 day the cells were harvested by centrifugation and washed three times with a physiological saline solution. Upon transferring them to a new environment, inoculum size was controlled to be approximately 3 \times 10^6 cells per ml.

**Measurements of cell growth and cellular division:** Cell growth was measured by turbidometry, and the degree of cellular division was expressed as the percentage distribution of cell lengths measured with about 3,000 individual cells.

**Staining:** Chromatin granules were stained by the method of Robinow (4) or a modification of that method, after fixing the cells with osmic vapour and hydrolyzing for 10 minutes with n-HCl at 60°. Gram staining was carried out by Hucker’s modified method (5).

**EXPERIMENTAL RESULTS**

1. *The influences of vitamin B_{12} upon cell growth and cellular division of L. delbrueckii*

   All cells grown in GYP-medium showed cell-length of less than 9μ (Fig. 1). These normal formed cells were harvested and transferred to the Difco’s B_{12} assay media containing increasing amounts of B_{12} for observation on the biological effects of the vitamin.

[Fig. 1. Distribution of cell length of organism cultured in GYP.]

[Fig. 2. Effect of vitamin B_{12} on growth of L. delbrueckii.]
Growth stimulation was distinct at a medium containing $5 \times 10^{-5}$ µg/ml of B12 and maximum growth was attained at a medium of $3 \times 10^{-4}$ µg/ml. In this range of concentration of the vitamin, the rate of cell growth was proportional to the amount of B12 in media, therefore, microbiological assay for this vitamin may be possible by applying this organism. (Fig. 2)

Cellular division, however, did not occur in parallel with cell growth. Namely, even at the concentration of $5 \times 10^{-4}$ µg/ml, at which maximum growth took place, the cells did not perform normal cellular division and the inhabitants of the medium were found to be a mixture of rods and filaments.

Stimulation of cellular division was observed gradually at a B12 content exceeding $10^{-2}$ µg/ml, and finally, at 1 µg/ml cellular division took place simultaneously with growth resulting in individual cell length of 3–6 µ (Fig. 3a, 3b, 3c, 3d). It is noteworthy that cell growth was almost constant within this B12 concentration range.

When cells were suspended in a medium containing only $10^{-3}–5 \times 10^{-4}$ µg/ml of B12, which is so dilute that both growth and division are extremely limited, typical cell elongation was occurred and frequently such cells as long as 300 µ could be observed (Fig. 3a). From these results it can be concluded that growth is not always followed by cellular division. Though vitamin B12 is essential for both growth and division of L. delbrueckii, a much higher concentration is required for division than growth. From the fact that such a high concentration of B12 is required for cellular division, each cell of L. delbrueckii ought to be considered to have the ability to accumulate approximately $10^5$ molecules of this vitamin.

For determining whether L. delbrueckii can retain B12 within cells, the
following experiments were carried out: cells propagated in GYP medium were thoroughly washed and inoculated in the B₁₂ assay media containing different amounts of B₁₂. Cells harvested from these media were washed and thrown again into the assay media without or with minute amounts of B₁₂.

The organism from a medium containing $5 \times 10^{-4} \mu g/ml$ B₁₂ could no more propagate in the absence of B₁₂, while cells from the medium containing $10^{-1} \mu g/ml$ of B₁₂ could grow normally even in the absence of B₁₂ (Fig. 4).

These results show that this strain not only requires B₁₂ as an essential growth factor but also has a tendency to take in and retain within its cell a much higher level of this vitamin than the amount indispensable for growth.

2. Chromatin structure

In higher plants and animals, nuclear division takes place prior to cell division. Numerous cytological observations on bacteria have also been amassed, however, so far no good agreement has been found among them on the behavior and functions of nuclei. The terminology of chromatin is used here for the granules which are highly basophilic and composed of deoxyribonucleic acid and assumed to be bacterial nucleus.

Chromatin granules of normal cells are of spherical or ellipsoidal configuration, and contained two per cell with 3μ in length and four with 6μ in length, i.e., the cell length per unit chromatin is 1.5μ. (pl. 1) Whereas, on the other hand, the granules in the elongated cell manifested typical compact aggregations resulting in cell length per one aggregate to be about 4.5μ. (Pl. 4). This clearly shows that abnormal nuclear division occurs in the filamentous cells.

3. Behavior toward Gram Staining

Gale (6) observed that Staphylococcus aureus, a typical Gram positive organism, inclined to Gram negative when cultured in a medium containing penicillin. In our investigation, L. delbruekii was shown to lose its Gram positivity in parallel with cell elongation. The longer elongated the cell, the paler it resulted in Gram staining (Pl. 3).

However, when the filamentous cells were treated with a medium containing a sufficient amount of vitamin B₁₂, they gradually recovered their Gram positivity, and thereafter divided into short segments which were undoubtedly the normal cells (Pl. 4).
4. **Plasmolysis and lysozyme treatment of filamentous cells**

Plasmolysis of the cells of *L. delbrueckii* occurred easily when cells were suspended in 0.5 molar solution of lactose or sucrose. The filamentous cells were washed and resuspended in 0.1-0.5 M lactose solution controlled to pH 6.8 with phosphate buffer. With increasing concentration of sugar, the rate of plasmolysis became higher, however, it always accompanied cells which did not plasmolyze at all. These peculiar cells lysed from the interior on treatment with lysozyme. (pl. 5). At the cells which had plasmolyzed, it could be observed that the contents of the cells divided into small condensed protoplasts, and the average cell length per unit protoplasm was about 4.5\(\mu\) (pl. 6). Each protoplasm in these cells easily changed to protoplast when the cell walls were eliminated by lysozyme.

**DISCUSSION**

It is well known that bacteriostatic agents such as some antibiotics induce the filamentation in bacteria. In *L. delbrueckii* a similar phenomenon had been observed with the cells grown in a magnesium deficient medium. However, cell elongation due to the deficiency of vitamin B\(_{12}\) may be representative among these phenomena.

As a rule, cell growth is considered to be always accompanied with cellular division. Lodge and Hinselwood (7), however, suggested that there was two independent factors controlling bacterial cell division and elongation respectively.

According to the opinion of the present authors, cellular division takes place only in a certain biological active state. Namely, unless an organism could attain this state, division could never occur. In the case of *L. delbrueckii*, this state of activity is considered to be accomplished in the presence of a high concentration of vitamin B\(_{12}\). And in the future, we intend to clarify how this highly active state be produced by vitamin B\(_{12}\). The filamentous cells are differed from normal cells by chromatin structure and Gram's reaction. The normal growing cells cultured in a medium containing 1 \(\mu\)g/ml of B\(_{12}\), clearly show a rhythmical cycle of elongation and division between cell lengths of 3\(\mu\) and 6\(\mu\), bearing 2 and 4 chromatin granules respectively. In contrast, the chromatin granules of filamentous cells are in a compactly aggregated state showing an abnormal figure of nuclei. Recently, Whitefield and Murrey (8) and further by Payne et al. (9) indicated that the chromatin aggregation is a secondary sequence due to the failure of homoeostatic mechanism regulating the ionic milieu within the cell. If this is the case, the deficiency of vitamin B\(_{12}\) is considered to deprive this mechanism from *L. delbrueckii*. Furthermore, it is important to consider the fact that the filamentous cells of *L. delbrueckii* are Gram negative. It is known that Gram dye retaining site is located near the surface of the cell and composed of magnesium ribonucleoprotein, and that for its synthesis, B\(_{12}\)
considered to be indispensable. Thus, the present authors wish to conclude that the primary sequence of B12-deficiency is to make the cell membrane abnormal. Abnormalities of cell surface, such as at the formation of transverse septa and in the synthesis of Gram's complex will be reasonably induced as secondary sequences. Gram negative filamentous cells having aggregated chromatin granules may thus be made to appear.

SUMMARY

A pleomorphic strain, *Lactobacillus delbrueckii* No. 1, requires vitamin B12 as an essential factor both for growth and division.

It has been determined that B12 concentration sufficient for growth (cell elongation) is $3 \times 10^{-4}$ µg/ml, while a concentration as high as 1 µg/ml is required for cellular division. This discrepancy is considered to be the cause of abnormal cell elongation of *L. delbrueckii*.

The elongated cells are also abnormal at Gram reaction and chromatin structure. These abnormalities have been concluded as the secondary sequences following to the disturbance of functions of cell surface (cytoplasmic membrane) caused by vitamin B12 deficiency.

REFERENCES

(4) C. F. Robinow: *J. Hygiene*, 43, 413 (1944).