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ANALYSIS OF A POPULATION OF Bacillus subtilis L-FORMS IN A FICOLL DENSITY GRADIENT

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UDC 576.8.095

The distribution of cells of a population of L-forms of Bacillus subtilis during isopycnic centrifugation in a Ficoll density gradient was analyzed. Two basic functions of the L-forms differing from one another in their buoyant density were distinguished. A study of the fractions by various methods showed that a high proportion of the population of L-forms consists of nonviable cells of different sizes, containing fragments of the genome or not containing DNA.

KEY WORDS: L-forms; density gradient; genome.

The results of phase-contrast and also of transmission and scanning electron microscopy have shown the well-marked polymorphism of L-forms [5, 6]. Members of the L-population differ not only in size, but also in submicroscopic structure and also, possibly, in methods of reproduction [1]. For this reason, separation of L-forms into fractions differing in biological properties is very interesting. The most widely used method for fractionation of L-forms, by filtration under pressure through filters with different pore diameters [9], yields fractions which differ only in the size of their L-forms. However, in this case the fractions are not even homogeneous in size. For instance, the absence of a rigid cell wall of the L-forms and their ability to undergo plastic deformation readily suggests that during filtration through pores, cells much larger than the diameter of the pores in the filters used are able to pass through [11].

In the investigation described below the L-population was analyzed by a method of separation based on buoyant density in a Ficoll density gradient.

EXPERIMENTAL METHODS

When the gradient material was chosen consideration was paid to the fact that it must not be toxic for L-forms, and the gradient produced must ensure isotonicity throughout its extent. This last property was essential so that the density of the cells would not change during centrifugation as they entered zones with different osmotic pressures of the medium. These requirements are satisfied by Ficoll-400 (from Pharmacia, Sweden), which has been used previously to study mycoplasmas [2, 3]. Separation in a Ficoll density gradient was carried out for different types of L-forms of B. subtilis, which were described as unstable, conditionally stable, and stable L-forms [4], in a Beckman L-65 centrifuge with SW50 rotor at 4°C for 5 h at 40,000 rpm. The NADH-oxidase activity of the separated fractions was determined by Pollack's method [8] and compared with the number of colony-forming units (CFU) and the DNA content (in cpm) in each fraction. For the electron-microscopic study of the fractions the generally accepted method of fixation, that of Ryter and Kellenberger, was used. The cells were dehydrated in alcohols and acetone and embedded in Araldite. The material was examined in the JEM-100B microscope with an accelerating voltage of 80 kV and instrumental magnification of 30,000 and 10,000×.

Laboratory of L-Forms of Bacteria, N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR P. A. Vershilova.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 88, No. 9, pp. 329-332, September, 1979. Original article submitted March 21, 1978.

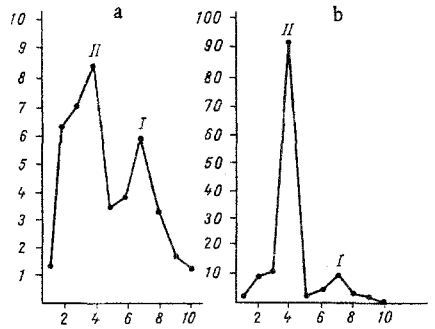

Fig. 1. Distribution of viable cells of a population of stable L-forms (a) and DNA of cells labeled with [^{3}H]thymidine (b) in a Ficoll density gradient. Abscissa, fraction Nos.; ordinate: a) log CFU, b) $^3H \cdot 10^3$ cpm. I) Peak corresponding to fraction I, II) peak corresponding to fraction II.

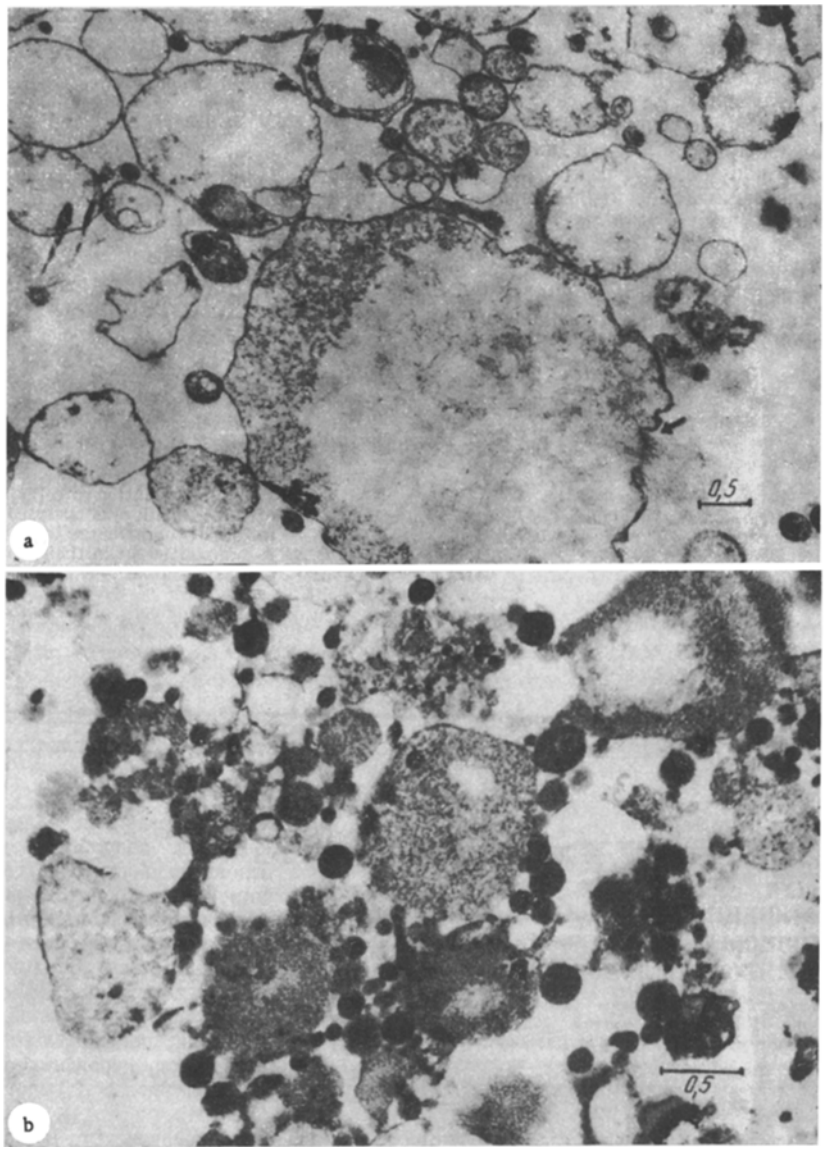

Fig. 2. Ultrathin sections of fractions of L-forms of B. subtilis: a) fraction I (16,000×); b) fraction II (28,000×). Arrow indicates extrusion of DNA fibrils from cell. Scale: 0.5 μ.

EXPERIMENTAL RESULTS

The character of distribution of the cells in the gradients was the same for L-forms of all three types indicated above. Two principal layers were formed in the population grown on liquid and solid medium. Phase-contrast microscopy showed that cells of the L-forms were present in both fractions. The mean density of the cells in them was 1.04 ± 0.01 and 1.10 ± 0.01 g/cm^3 respectively.

Analysis of the distribution of viable cells (CFU) and of DNA of cells labeled with [^{3}H]thymidine in the density gradient showed the existence of two peaks corresponding to layers I and II (Fig. 1). The number of viable cells in the top fraction I was below 1% of the number of viable cells in the bottom fraction II (more than 95% of all viable cells of the population were present in fraction II). The DNA content in fraction I was about 10% of the DNA content in fraction II. Despite such substantial differences between the fractions for the number of CFU and the DNA content, the turbidity of the fractions measured spectrophotometrically was of the same order. In the writers' view, the turbidity of fraction I reached this high value on account of its nonviable L-cells, with an incomplete genome. If this suggestion is correct, the ratios of membrane structures/DNA and membrane structures/CFU in fraction I ought to be much greater than those for fraction II, in which viable cells predominated.

To compare the membrane content in the fractions their NADH-oxidase activity was measured, for this is a marker enzyme of bacterial membranes [8]. If the NADH-oxidase activity of fraction I was only twice the activity of fraction II, the ratio for the DNA concentration would be more than one order of magnitude greater, and for the number of CFU it would be 10^3 times greater.

Electron-microscopic studies showed that fraction I contains spherical or irregularly shaped cells measuring from 0.2 to 5 μ, totally without contents or with cytoplasm of very low density. Most cells had no nucleoid. In the few cells which had a nucleoid, discharge of DNA fibrils into the surrounding medium through the damaged membrane could be observed (Fig. 2a). In other words, judging from the morphological picture, most of the cells in this fraction were nonviable.

Fraction II also contained cells of widely different diameters (from 0.1 to 1.5 μ). By contrast with the previous fraction, however, most cells had a well defined cytoplasm in which ribosomes and a clearly outlined nucleoid with DNA fibrils could be seen (Fig. 2b). Only in the smallest cells (measuring from 0.13 to 0.25 μ) were ribosomes and DNA fibrils not visible. The reason could be the extremely high density of the cytoplasm.

The electron-microscopic results thus confirmed the suggestion that the fractions differ in the viability of the cells present in them. The peak of viability in fraction I can be explained by the presence of large bodies with a complete genome; because of their greater volume, the large bodies have a different buoyant density from the majority of viable cells. The tendency of L-forms to form conglomerates may be another explanation. One or two viable cells may "stick" in the conglomerates of nonviable cells, giving rise to the peak of CFU in fraction I.

The results show that the formation of L-forms not containing a complete genome is a characteristic feature of the population of L-forms. Electron-microscopic data show that the nucleoids was absent not only in elementary particles under 0.25-0.3 μ in diameter, the viability of which has frequently been discussed [7, 10], but also in the considerably larger spherical or irregularly shaped cells.

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