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EXPERIMENTAL ARTICLES

Research on the Heterogeneity of a *Micrococcus luteus* Culture during an Extended Stationary Phase: Subpopulation Separation and Characterization

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Abstract—After prolonged starvation, a population of stationary-phase *Micrococcus luteus* cells (containing dormant cells, as shown earlier) was investigated using a flow cytometer and the method of distributing cells in a two-phase system composed of aqueous solutions of polymers. Flow cytometry revealed the existence of two cell subpopulations distinguished by their ability to bind the fluorescent probe rhodamine 123. In the two-phase system, the two subpopulations were located in the polyethylene glycol (PEG) phase at the interface. The cell subpopulation with enhanced fluorescence, isolated with a cell sorter, contained viable (colony-forming) cells and dormant cells which could be resuscitated. Most viable cells stayed in the PEG fraction. Electron microscopy showed that the PEG fraction predominantly contained intact cells.

Key words: Micrococcus luteus, dormant forms, population heterogeneity.

The dormant (anabiotic) state can be defined as a reversible state with low metabolic activity in which cells can persist for a long time without dividing [1]. Recently, extensive research data have been obtained which suggest that nonsporulating bacteria (like sporulating bacteria) can assume a dormant state [2–5]. In many cases, such cells are unable to form colonies on solid media. However, these nonculturable forms can presumably be converted to colony-forming (CF) viable cells through a resuscitation procedure [1, 4, 5]. Possibly, the dormant state is characteristic of such human-endangering bacteria as *Mycobacterium tuberculosis* [6], *Vibrio cholerae* [7], *Salmonella* spp. [8, 9], and *Helicobacter pylori* [10]. The problem is of importance both for medical microbiology and ecological safety–related research.

We established in our works that the dormant state can be attained by cells of the nonsporulating copiotrophic gram-positive coccus *Micrococcus luteus* starved for 3–6 months after reaching the stationary growth phase. When inoculated on petri dishes, this culture is characterized by a low number of viable cells ($<10^4$ cells/ml), whereas the total cell number is close to its original value [1]. Using a flow cytometer and suitable probes under appropriate experimental conditions [11], we revealed that a significant part of the cells of a *M. luteus* culture starved for 3 months can be converted into CF forms [3]. Our findings were confirmed by the serial dilution method; in these studies, the resuscitation procedure was accomplished in a medium which, in statistical terms, contained no viable cells [12]. The addition of sterile filtrate of a log-phase *M. luteus* culture was prerequisite for successful cell resuscitation [12]. Along with other related data [13, 14], these results allowed us to conclude that a significant portion of cells in a starving *M. luteus* culture are dormant in physiological terms. The percentage of dormant cells varied between 10 and 90% of the population in different experiments. However, these estimates could only be made after resuscitating the cells. The point to be clarified concerned the differences among the individual cells of a starving population which determine their fate during the resuscitation procedure. Solving this problem required separating the culture into subpopulations containing cells with physiologically different properties. The separation procedure was the focus of this work.

MATERIALS AND METHODS

Cultivation of bacteria and the starvation conditions. This work used a culture of *Micrococcus luteus*, strain NCIMB 13267, grown aerobically at 37°C with vigorous shaking in flasks in minimum medium with lactate as the carbon source, as described earlier [15]. Lactate, sterilized by passing through filters with a pore size of 0.22 μm, was added to the medium immediately before the inoculation. After reaching the stationary phase, the shaking of the culture was continued for

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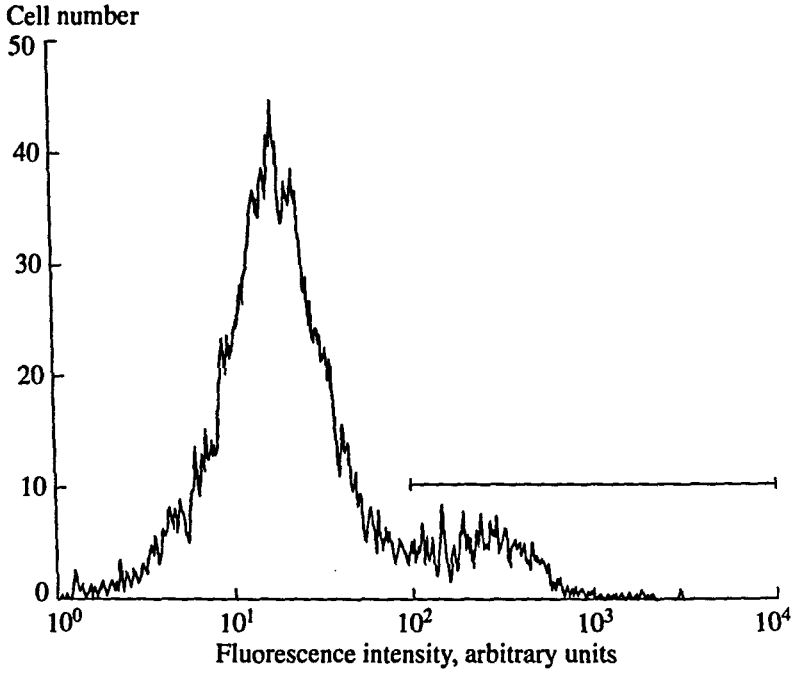

Fig. 1. Distribution of *M. luteus* cells stained with rhodamine 123 in terms of fluorescence intensity, which was measured by an Elite flow cytometer. The cells were starved for 5 months. Before the measurements, the culture was 20-fold diluted with minimum medium without lactate. The photomultiplier voltage for the fluorescence channel was 700 V. The horizontal line is the boundary of zone B (see text).

2 months at 30°C. Subsequently, the culture was stored for 2–4 months at room temperature.

Analysis and separation of cell populations with a flow cytometer and a cell sorter. Individual cells in a population were analyzed using the Cultur EPICS Elite flow cytometer–sorter. Cells were stained with the fluorescent dye rhodamine 123 (at a final concentration of 0.3 μM). To reduce cell aggregation, all samples were thoroughly suspended by passing them through a syringe with a needle diameter of 0.4 mm. All measurements were done according to the manufacturer's manual. Excitation light with a wavelength of 488 nm was emitted by an argon laser (15 mW) with an air cooling system. Cells were sorted under the following conditions: the drop frequency was 18 kHz, the photomultiplier voltage was 400 and 800 V for measuring light scattering and rhodamine 123 fluorescence, respectively. The flow medium contained 150 mM KCl and 10 mM K-HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 6.8). The cell count rate did not exceed 100 cells per sec.

Separation of cell cultures in a two-phase system was done using a mixture of 5% dextran T500 and 5% polyethylene glycol (PEG) 6000 in 10 mM potassium phosphate buffer (pH 7.0). The whole separation procedure was carried out under sterile conditions. The PEG/dextran suspension was passed through a Millipore or Gelman bacterial filter with a pore size of 0.22 μm. Starving cells were washed with sterile phosphate buffer and suspended in a small volume of this

Table 1. Distribution of the total cell population and of colony-forming (CF) cells of a *M. luteus* culture starved for 3 months between the fractions obtained by separation with a cell sorter and the viability rates in the fractions

Fraction	CF cells, % of the total CF cell number in all fractions	Cells, % of the total cell count in all fractions	Viability rate in the given fraction, %	Ratio of the viability rate in the given fraction to the viability rate in the original culture before separation
Zone A	0.67	80	0.0053	0.3
Zone B	99.3	20	3.1	155
Before separation	–	–	0.02	–

Note: Viability rate is defined as the ratio between the number of CF cells and the total cell count.

buffer. A dense suspension was applied to a PEG/dextran mixture and vigorously drawn 10–15 times through a thin-needle syringe. The mixture was then incubated for 1.5–2 h at room temperature (20–22°C) to achieve phase separation. The separated phases were carefully collected with a syringe.

Fluorescent microscopy. A Leica (LEITZ DM) epifluorescent microscope with filter block no. 13 (excitation at 450–490 nm) was used to record the fluorescence of cells stained with rhodamine 123.

Electron microscopic studies were conducted as described earlier [13].

Cell viability was estimated by two methods: by inoculation on petri dishes with solid medium and by the serial dilution method. In the former method, the agar medium contained 1.3% Lab M nutrient broth E. Serial tenfold dilutions were prepared in minimum medium without lactate. Before inoculation, cells were thoroughly suspended, as described above, in order to prevent cell aggregation. The petri dishes were incubated at 30°C for 3–5 days. Determination of viability by the serial dilution method was made with the use of a Bioscreen C optic analyzer (Labsystems, Finland). Thoroughly suspended cells were diluted as described earlier [12]. 10-μl portions of each dilution were placed in wells containing 200 μl of either minimum medium with 0.5% lactate and 0.05% yeast extract or the same medium diluted at a ratio of 1 : 3 with a *M. luteus* culture supernatant obtained as described earlier [12] and passed through a 0.22-μm-pore-size filter. The plates were incubated at 30°C with vigorous shaking. The total incubation time was 120 h, and the measurements were done once an hour. Viable cell numbers were calculated using the tables presented by Collins and Lyne [16].

Total cell numbers were determined in a Goryaev chamber [15].

Reagents. We used Sigma rhodamine 123, Merck PEG 6000, and Loba Feinchemie dextran T-500. Other reagents used by us were obtained from Sigma or BDH or produced in Russia ("chemical purity grade" or "special purity grade").

RESULTS

Figure 1 demonstrates the distribution of *M. luteus* cells in a flow cytometer after staining the cells with the positively charged probe rhodamine 123. The culture used in our experiments stayed in the stationary stage for three months. It is evident that at least two cell populations with different fluorescence intensity (zones A and B) occur. It should be noted that, in this system, probe fluorescence reflected passive probe binding (in contrast to active micrococcal cells from the logarithmic phase [3]), since adding the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) did not alter the distribution pattern (data not shown).

The starved culture was separated into two subpopulations, corresponding to zone A and zone B, with a cell sorter. After the separation procedure, the cells from each zone were inoculated onto agar medium to determine the number of CF cells; the total cell numbers were also counted. Incubation of both starved and freshly grown cells for 2 h in the buffer used in the cell sorter did not affect their viability (data not shown). Table 1 presents the results of a typical experiment. The overwhelming majority of the CF cells turned out to belong to zone B (over 99%), where a viable subpopulation made up 3.1%. The portion of CF cells in population A was only 0.007%. The total cell numbers in zones A and B were at a ratio of 8 : 2.

The other approach to the separation of a starved culture into subpopulations used a two-phase system of polymer (PEG and dextran) solutions, which provides for separation of particles with different surface properties such as hydrophobicity and surface charge. According to the data available in the literature, the distribution pattern in such systems depends on the ion composition of the medium [17]. To separate a starved *M. luteus* culture, conditions were created (see "Materials and Methods") under which the cells were chiefly distributed between two fractions: the PEG-enriched upper phase and the phase boundary (the interface). The quantitative data on the distribution pattern are given in Table 2.

Table 2. Distribution of the total cell population and of colony-forming (CF) cells of a *M. luteus* culture starved for 4–6 months between the phases of the two-phase system and the viability rate in the phases

Fraction	CF cells, % of the total CF cell number in all fractions	Cells, % of the total cell count in all fraction	Viability rate in the given fraction, %	Ratio of the viability rate in the given fraction to the viability rate in the original culture before separation
PEG	67	23	0.26	13
Interface	32	75	0.06	3
Dextran	>1	2	0.02	~1
Before separation	–	–	0.02	–

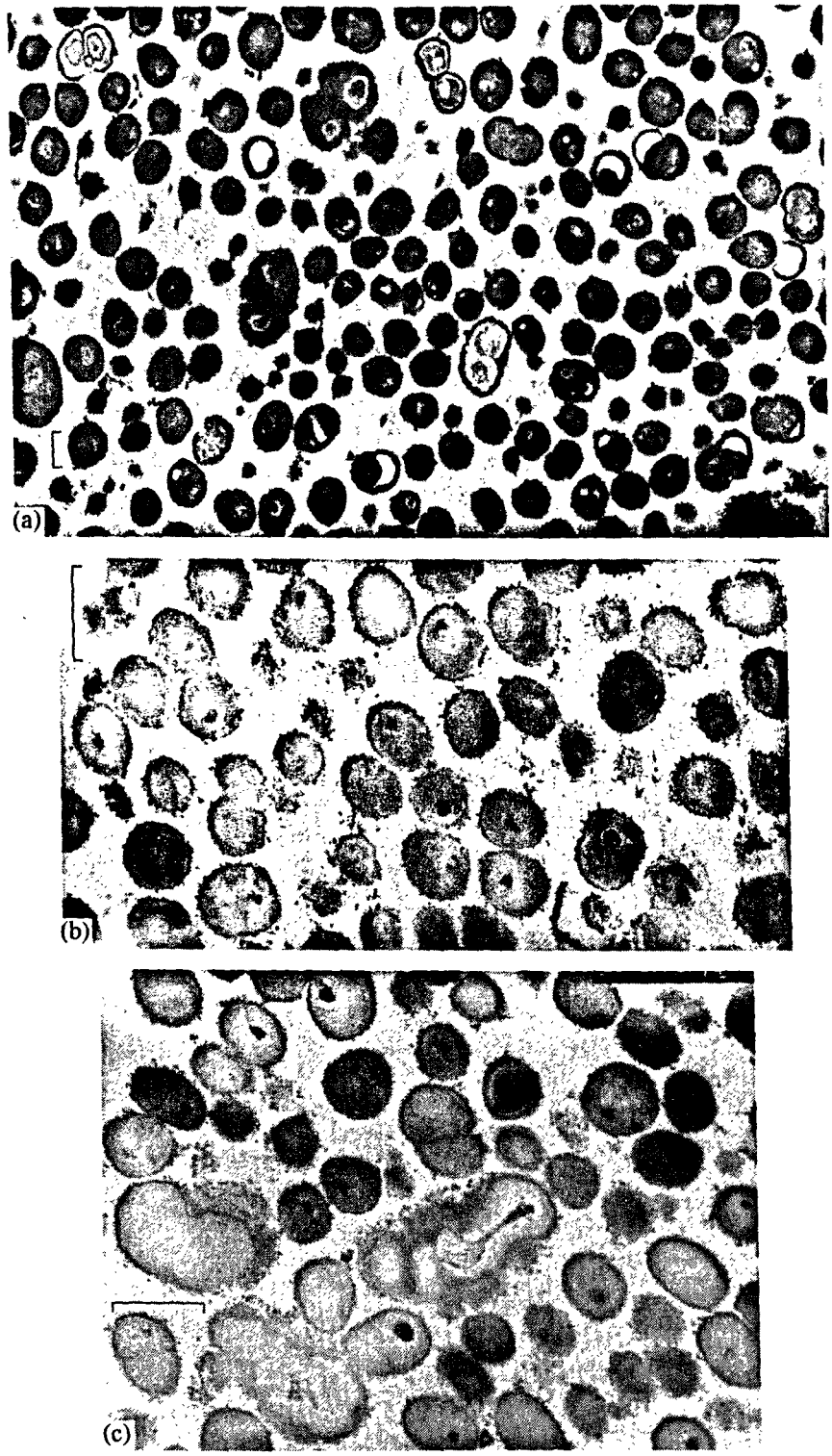

Fig. 2. Electron micrographs of ultrathin sections of a *M. luteus* culture starved for 5 months. (a) The original culture; (b) and (c) the subpopulations obtained by separation in the two-phase system (b and c show PEG fraction and interface cells, respectively). The bars represent 1 μm.

It is evident that the CF cells tended to concentrate in the upper (PEG) fraction: the cell viability in the upper phase and at the interface was 0.26% and 0.06%, respectively. Interestingly, the total CF cell number after separation is several times greater than that before separation. In control experiments, cell suspensions

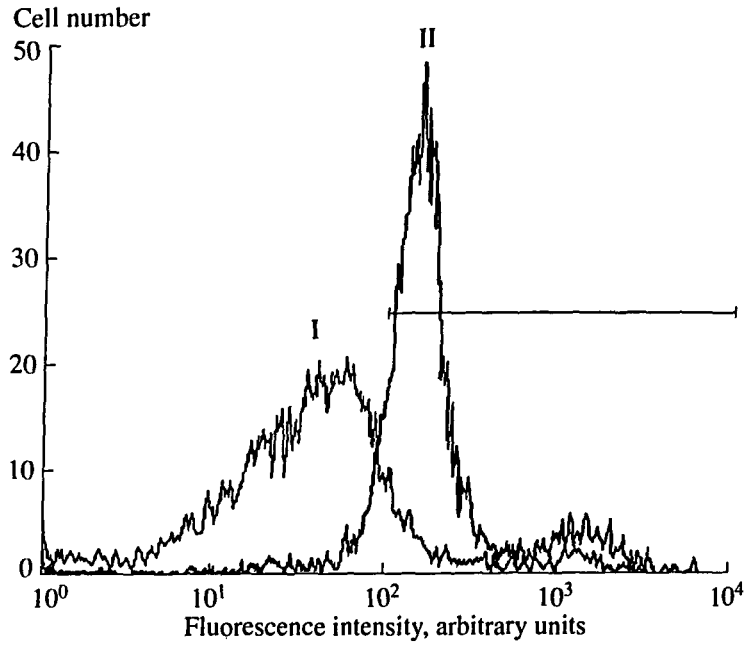

Fig. 3. Distribution of *M. luteus* cells stained with rhodamine 123 in terms of fluorescence intensity, which was measured by an Elite flow cytometer. The cells, starved for 5 months, were separated in the two-phase system (see "Materials and Methods"). Aliquots of the PEG and interface fractions were 10-fold diluted with minimum medium without lactate. (I) Cell distribution in the interface fraction; (II) cell distribution in the PEG fraction. For other conditions, see Fig. 1 caption. The horizontal line is the zone B boundary (see text).

from an unseparated PEG/dextran mixture were inoculated, and only an 1.5-fold increase in cell viability was found.

Electron microscopy was used to establish the structural peculiarities of the cells of each of the two phases. In Fig. 2a, an electron micrograph of the original, unseparated *M. luteus* culture starved for 4 months is presented. The culture is obviously heterogeneous, with several cell types differing in size and structure. The majority of the cells is characterized by a size close to that of log-phase cells (0.6–0.8 μm) [13], but the cell walls are thicker and more electron-dense, and the nucleoids are compact. Another cell type is bigger in size (1.3–1.5 μm). The thin cell walls are frequently characterized by uneven thickness and the large nucleoids have a coarse globular structure. Apart from these two types, the culture contains a number of lysed cells (about 0.8 μm in size). All the cells of the starved population lack the mesosomal membrane structures invariably occurring in log-phase cells in the vicinity of the nucleoid [13].

After separating the culture in the two-phase system, relatively homogeneous cells occurred in the upper phase, with rare cells (0.6–1.2%) of increased size (Fig. 2b). Cells with signs of degradation were also rare (1.7%). From Fig. 2c, it follows that considerable heterogeneity is characteristic of the cells at the interface. The percentage of large and degraded cells was 3 and 13%, respectively; in some microscope fields, degraded cells accounted for 1/4 or 1/5 of the total cell number. In total, 38 and 34 microscope fields were analyzed for samples from the upper phase and the interface, respectively, at a magnification of 11 000× to 18 000×. The samples were taken from four separation experiments.

Flow cytometry of rhodamine 123–stained cell subpopulations obtained in the two-phase system revealed significant differences between them (Fig. 3). The cells concentrating in the PEG fraction demonstrated a comparatively narrow distribution pattern of fluorescence intensity and were distinguished by a higher fluorescence intensity in comparison with the interface cells. The interface cells also showed the bimodal distribution pattern characteristic of unseparated cells and the PEG-fraction cells, but this pattern was less clearly manifested.

DISCUSSION

Employment of two independent experimental approaches enabled us to demonstrate the heterogeneity of the *M. luteus* cell population after an extended stationary phase and to obtain subpopulations enriched in different cell types.

Despite the different principles used for separating cells (flow cytometry based on rhodamine 123 binding by cells and cell distribution in a two-phase system), the resulting subpopulations are likely to contain similar cell types. This follows from the data on the viable

cell number in the fractions of the two-phase system and in the distribution zones in the flow cytometer (Table 1 and 2). In addition, the starved cell population contained two cell types with high and low fluorescence intensity after staining with rhodamine 123 (maximum fluorescence intensity of the first and second phase was 20 and 250–300 arbitrary units, respectively). Similar subpopulations were obtained in a two-phase system (maximum fluorescence was 40 and 200 arbitrary units) (Fig. 3). It was the cell population with increased fluorescence that was characterized by enhanced viability. The part of the curve with fluorescence values over 1000 units seems to correspond to the cells with increased size.

Spores of microorganisms are characterized by a relatively hydrophobic surface [18], and they tend to concentrate in the PEG fraction of the two-phase system. Presumably, the surface of the *M. luteus* cells surviving under unfavorable environmental conditions is more hydrophobic than the surface of the unviable cells. Enhanced fluorescence after rhodamine 123 binding to zone B or PEG fraction cells is additional evidence in favor of this suggestion (Figs. 1, 3). Many nonsporulating bacteria are known to adapt to starvation by increasing the hydrophobicity of their surface [1]. It follows that the dormant cells should concentrate in zone B or in the PEG phase, where most CF bacteria are also located.

If this is the case, then the number of CF cells should increase in both aforementioned subpopulations after resuscitation procedures, a fact actually established for the zone B cells after sorting the cells with a flow sorter [19]. However, resuscitation of the PEG fraction cells failed to yield a similar result (data not shown), which is plausibly due to the considerably lower separation efficiency of this method (a 13-fold viability increase in the PEG fraction in contrast to a 155-fold viability increase in zone B). Possibly, the presence of a significant number of unviable cells in the PEG fraction hampers the successful resuscitation of the dormant cells of this subpopulation due to the release of some growth inhibitors by the unviable cells [20]. Electron microscopy also confirms the conclusion that the intact cells are preferentially located in the PEG fraction. The cells with increased size occur both in the PEG phase and at the interface. They are more abundant in the interface. The cells with increased size are, therefore, unlikely to be capable of forming colonies. Probably, they represent unviable cells characterized by unbalanced growth.

Thus, the population of the nonsporulating bacterium *M. luteus* starved for 3–6 months is heterogeneous, consisting of viable, dormant, and unviable cells, which can influence each other during the resuscitation process [14, 20]. The viable and dormant subpopulations can be separated from the unviable cells by a method based on their ability to bind the weakly lipophilic cation rhodamine 123. Cell separation with a cell sorter is a promising method in terms of the analysis of the heterogeneity of bacterial systems [21, 22], although this method does not yield sufficiently large numbers of sorted cells for their subsequent analysis. The two-phase system, which makes it possible to separate preparative amounts of various types of bacterial cells [17, 23], is more advantageous in this respect (despite its lesser selectivity).

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