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Quantitative Analysis of the Physiological Heterogeneity within Starved Cultures of *Micrococcus luteus* by Flow Cytometry and Cell Sorting

ARSENY S. KAPRELYANTS,[1] GALINA V. MUKAMOLOVA,[1] HAZEL M. DAVEY,[2] AND DOUGLAS B. KELL[2]*

Institute of Biological Sciences, University of Wales, Aberystwyth, Dyfed SY23 3DA, United Kingdom,[2] and Bakh Institute of Biochemistry, Russian Academy of Sciences, Leninskii Prospekt 33, Moscow 117071, Russia[1]

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A high proportion of *Micrococcus luteus* cells in cultures which had been starved for 3 to 6 months lost the ability to grow and form colonies on agar plates but could be resuscitated from their dormancy by incubation in an appropriate liquid medium (A. S. Kaprelyants and D. B. Kell, Appl. Environ. Microbiol. 59:3187–3196, 1993). We used flow cytometry and cell sorting to study populations of bacteria that had been starved for 5 months. These cells could be stained by the fluorescent lipophilic cation rhodamine 123, but such staining was almost independent of metabolically generated energy in that it was not affected by uncouplers. Two populations could be distinguished, one with a lower degree of rhodamine fluorescence (a degree of fluorescence referred to as region A and containing approximately 80% of the cells) and one with a more elevated degree of fluorescence (region B, approximately 20% of the cells). Subsequent incubation of starved cells in fresh medium in the presence of the antibiotic chloramphenicol (to which *M. luteus* is sensitive) resulted in the transient appearance of cells actively accumulating rhodamine 123 (and fluorescing in region B) and of larger cells exhibiting a yet-greater degree of fluorescence (region C). These more fluorescent cells accounted for as much as 50% of the total population, under conditions in which the viable and total counts were constant. Thus, metabolic resuscitation of at least one-half of the cells takes place under conditions in which cryptic growth cannot play any role. Sorting experiments revealed that the great majority of the viable cells in the starved population are concentrated in regions B and C and that the extent of rhodamine staining under conditions of starvation therefore reflects the physiological state of the cells. Physical separation of these cells from cells in region A resulted in an increase (of approximately 25-fold) in the viability of cells in regions B and C and of the population as a whole. Resuscitation of dormant cells in a most-probable-number assay in the presence of supernatant taken from growing *M. luteus* revealed the resuscitation of cells from regions B and C but not from region A. It is suggested that initially dormant (resuscitable) cells are concentrated in regions B and C.

Dormancy may be defined as "a reversible state of low metabolic activity, in which cells can persist for extended periods without division" (4); in microbiology this often corresponds to a state in which cells are not alive in the sense of being able to form a colony when plated on a suitable solid medium but in which they are not dead in that when conditions are more favorable they can revert, by a process known as resuscitation, to a state of aliveness as so defined. The possible existence of dormant forms therefore has profound implications for public health microbiology and for our understanding of microbial behavior generally.

In recent work, we have found that cells of the nonsporulating, copiotrophic, gram-positive coccus *Micrococcus luteus* starved for 3 to 7 months in spent growth medium following growth to stationary phase in batch culture can persist in a dormant state in cultures which overall exhibited a very low level of viability ($<10^{-4}$) as estimated by plating on agar plates, while the total count remained close to its initial value (4, 6). Using flow cytometry with appropriate probes and conditions (9), we found that a high percentage of *M. luteus* cells in 3-month-old populations could be resuscitated to normal, colony-forming bacteria (6), under conditions which excluded any significant regrowth of initially viable cells. We confirmed this by using the most-probable-number (MPN) method, when we resuscitated cells in media which, statistically, contained no initially viable cells (7). These and other data (12, 24) led us to the conclusion that from a physiological point of view, a significant number of cells in such starved *M. luteus* populations were not dead but were dormant and could be converted to normal, colony-forming bacteria. This percentage of dormant bacteria has been estimated to be around 50% for bacteria starved for some 75 days, the overwhelming bulk of the other 50% of the cells being in a physiological state indistinguishable from death (6). However, the assessment of the percentage of resuscitable cells in a population could, on the basis of these and related experiments (5), only be made after cell resuscitation when, under some conditions, an excellent correlation between the percentage of dormant cells and the different extents to which rhodamine 123 (Rh123) could be accumulated in an uncoupler-sensitive fashion by different cohorts of cells could be found. A particularly desirable goal would therefore be to establish which differences between the individual cells in a population before resuscitation can be correlated with whether such cells survive.

To this end, in the present study we used flow cytometry and cell sorting procedures, which allow one to analyze cell populations by flow cytometric procedures and then to physically separate subpopulations of cells with different properties for their further examination (see, e.g., references 22 and 23). It is therefore necessary to develop appropriate criteria (and appropriate probes) which would allow one to distinguish and therefore separate cells in the different physiological states of interest. We here describe the use of the cationic dye Rh123 to monitor differences not only between metabolically active and

* Corresponding author. Mailing address: Institute of Biological Sciences, University of Wales, Aberystwyth, Dyfed SY23 3DA, United Kingdom. Phone: 44 1970 622334. Fax: 44 1970 622354. Electronic mail address: DBK@ABER.AC.UK.

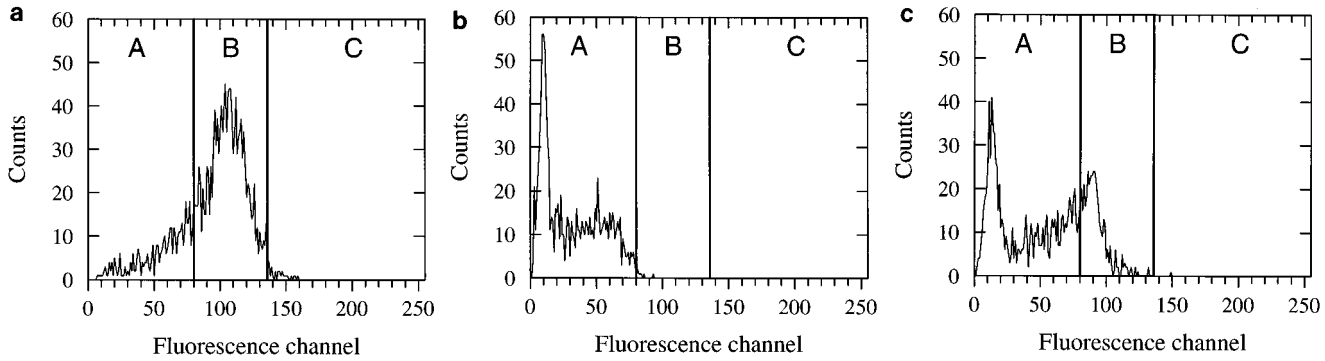

FIG. 1. Distribution of the fluorescence of nonstarved (a and b) and starved (c) cells of *M. luteus* stained with Rh123 and assessed by flow cytometry. Cultures were grown in lactate minimal medium until late logarithmic phase, harvested, washed, resuspended in lactate minimal medium lacking lactate, and stained with Rh123 (0.3 μM) (a and b) or starved for 5 months and diluted 20-fold in lactate minimal medium without lactate. The distribution of fluorescence was assessed by analytical flow cytometry with the Skatron Argus 100 instrument, and cells were analyzed for the ability to accumulate Rh123 as described in Materials and Methods. For cells represented in panel b, 15 μM CCCP was added to the cells before measuring was done. The photomultiplier tube voltage for the fluorescence channel was 700 V, and the full scale of the abscissa represents 3.5 decades in fluorescence intensity (2). The percentage distribution of cells is as follows: (a) A, 21%; B, 78%; C, 1%; (b) A, 98%; B, 2%; (c) A, 80%; B and C, 20%.

inactive cells during starvation but also between such cells at the end of the starvation period before resuscitation.

We previously found that during the initial steps of resuscitation an important early step was the restoration of the membrane permeability barrier as assessed by flow cytometry with the normally membrane-impermeant probe 4-(3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylidene)-1-(3′-trimethylammonium propyl)-pyridinium diiodide (PO-PRO-3) (24). To minimize the probability of any involvement of the regrowth of a small number of "initially viable" cells during resuscitation, we used pencillin pretreatment and cell dilution to decrease the concentration of viable cells at the beginning of resuscitation (6, 24). At the same time, we found that the presence of a small fraction of viable cells at the onset of resuscitation facilitates the recovery of the remaining dormant cells (24). The possible influence of cryptic growth has been found for resuscitation of so-called viable but nonculturable *Aeromonas salmonicida* (11, 20) and *Vibrio vulnificus* (3, 15, 16, 25). We therefore devised protocols for resuscitation in which influence of cryptic growth could be excluded with certainty while the population still contained the same proportion of viable cells.

MATERIALS AND METHODS

Organism and media. *M. luteus* NCIMB 13267 (previously described as Fleming strain 2665) was grown aerobically at 30°C in shake flasks in lactate minimal medium containing L-lactate as described previously (5), except that the lactate was sterilized by filtration through a 0.22-μm-pore-size filter. When the culture had reached stationary phase, agitation was continued at 30°C for up to 3 months. Cultures were then held in closed bottles at room temperature without agitation for a period of up to a further 2 months.

Cell recovery after starvation. Starved cells were inoculated, to a cell density of some $2 \cdot 10^8$ to $2.5 \cdot 10^8 \cdot ml^{-1}$, into fresh lactate minimal medium containing 0.5% (wt/vol) L-lactate, 0.05% yeast extract, or no added nutrients and appropriate antibiotics as indicated in the figure legends. The cell cultures were incubated aerobically at room temperature without agitation. Periodically, samples were withdrawn to check microbiological purity and total and viable counts and for flow cytometry.

Analytical flow cytometry. The flow cytometric analysis of the distribution of cells in populations in experiments using Rh123 uptake was performed exactly as described elsewhere (5) by using a Skatron Argus 100 instrument. To stain cells with PO-PRO-3, we used a protocol described earlier (24). All samples were carefully dispersed before flow cytometry by repeated extrusion through a 0.4-mm-diameter hypodermic needle to eliminate clumps of organisms.

Flow cytometric cell sorting. Measurements were performed by using a Coulter EPICS Elite cell sorter according to the manufacturer's instructions. Excitation was done with an air-cooled 15 mW Ar^+ laser at 488 nm, and measurements were gated on the forward-scatter channel. Sorting was carried out under the following conditions: frequency of droplet formation, 18 kHz; deflection amplitude, 100%; 3 drops sorted per event; drop delay, 21 drops. Detector voltages were 400 and 800 V for forward scatter and Rh123 fluorescence, respectively. The sheath fluid consisted of 150 mM KCl–10 mM K-HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) (pH 6.8). The data rate was maintained below 100 counts $\cdot s^{-1}$.

Fluorescence microscopy. A Leica epifluorescence microscope (LEITZ DM RB) with an I3 filter block (excitation, 450 to 490 nm; band stop, 510 nm; emission, >520 nm) was used to monitor the fluorescence of cells stained with Rh123.

Cell viability by plating. Plates containing 1.3% nutrient broth E (Lab M) or lactate minimal medium were used. Cell dilutions were made in quadruplicate with lactate minimal medium lacking lactate. No differences in viable counts were found if rich medium (nutrient broth E) instead of lactate minimal medium was used for cell dilutions. Cells were carefully dispersed by repeated passage through a 0.4-mm-diameter needle to avoid clumping. Plates were incubated at 30°C for 3 to 5 days.

Cell viability by MPN. The MPN assay was performed in a Bioscreen C optical growth analyzer (Labsystems, Helsinki, Finland). Cells were first carefully dispersed, and dilutions of each fraction tested were made as described elsewhere (7). A 10-μl volume of each dilution (five replicates) was added to a well containing 200 μl of either lactate minimal medium supplemented with 0.5% lactate and 0.05% yeast extract or the same medium diluted (1:3) with supernatant taken from growing *M. luteus* prepared as described elsewhere (7). A wide-band filter was used to monitor the optical density. Plates were incubated at 30°C with "intensive continuous" shaking (set by the instrument's software). The total measurement time was 120 h, each well being measured hourly. The calculation of the MPN was based on published tables (1).

Total cell counts. Unstained cells were counted with a phase-contrast microscope and an improved Neubauer counting chamber (5).

Chemicals. Rh123, penicillin G, chloramphenicol, and cerulenin were obtained from Sigma, Poole, United Kingdom. PO-PRO-3 was obtained from Molecular Probes, Eugene, Oreg. Other chemicals were of analytical grade and were obtained from Sigma or BDH.

RESULTS

Heterogeneity of starved *M. luteus* cell cultures and its change during cell recovery. We have described elsewhere the procedure for the resuscitation of extensively starved *M. luteus* cells which involved incubation of the cells in fresh liquid medium (6, 24). In those studies, starved cells were incubated after dilution in lactate minimal medium in the presence of penicillin to decrease the number of viable cells present at the onset of resuscitation, and we used flow cytometry and the probe Rh123 as an indicator of the restoration of active metabolism in cells. However, in the previous work the antibiotics were removed during the resuscitation phase.

First we made a detailed study of the distribution of cells in nonstarved and starved *M. luteus* populations by flow cytometry. Figure 1a and b shows the typical distribution of the fluorescence of nonstarved *M. luteus* cells that had been

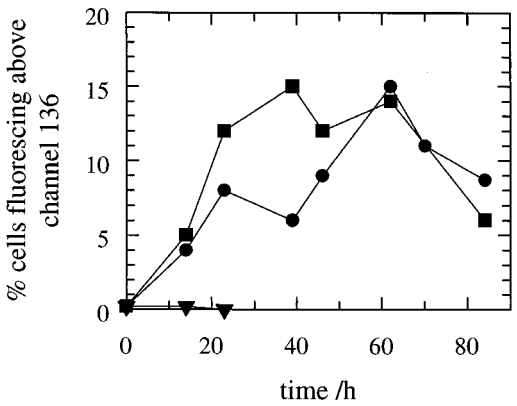

FIG. 2. Changes in the percentage of cells accumulating Rh123 in region C shown in Fig. 1 in starved *M. luteus* during incubation in fresh medium with chloramphenicol. The experiment was performed as described in the legend to Fig. 1 and Materials and Methods. The media were lactate minimal medium lacking lactate but with no other added nutrients (circles), the same medium with 0.05% yeast extract (triangles), and the same medium with 0.5% Li lactate (squares).

stained with Rh123 and studied in the flow cytometer. We discriminated the extent of staining as follows. Channel 136 (which corresponds to a particular intensity of fluorescence of a particle observed by the fluorescence detector) was chosen as a threshold since almost no Rh123-stained, starved cells exhibited a fluorescence in a channel greater than this, and freshly harvested, viable cells in the presence of Rh123 and the absence of uncoupler exhibited a fluorescence between channels 80 and 136 (Fig. 1a), which is fully uncoupler sensitive (Fig. 1b). Figure 1c shows a typical distribution of the fluorescence of *M. luteus* cells that had been starved for 5 months, stained with Rh123, and studied by flow cytometry. A bimodal distribution in the extent of staining is evident. Region A (channel 0 to channel 80) represents cells which bind Rh123 nonspecifically: 98% of fresh late-logarithmic-phase *M. luteus* cells stained with the same concentration of Rh123 and then treated with a suitable concentration of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) exhibited a fluorescence in this region (Fig. 1b). This influence of CCCP was not due to simple fluorescence quenching, as has been shown previously (5). Although starved cells in region B (between channels 80 and 136) shown in Fig. 1c had an elevated fluorescence relative to that of those in region A, its sensitivity to CCCP was also very low (only 2 to 5% of the cells in region B exhibited a decrease in fluorescence after CCCP treatment). This phenomenon was not due to any inability of the uncoupler to act per se, since octanol treatment also failed to decrease the extent of staining of such cells.

For the different antibiotics tested to ensure the avoidance of cryptic growth, which included penicillin, novobiocin, chloramphenicol, and cerulenin, we found a transient increase in the number of cells accumulating Rh123 cells in regions B and C during incubation in fresh medium in the presence of chloramphenicol (and of cerulenin). Figure 2 shows the increase of Rh-accumulating cells in region C (above fluorescence channel 136) both in unsupplemented medium and in minimal medium supplemented with lactate or yeast extract. The maximum percentage of cells exhibiting this degree of fluorescence occurred after 40 to 60 h of incubation of the starved cells in resuscitation medium, while after 80 h the proportion of such cells decreased. The fluorescence of cells that appeared in region C was essentially completely sensitive to uncoupler (less than 0.5% of the cells were left in this region after incubation with CCCP). Flow cytometry also revealed the accumulation of larger cells among the cells in region C (data not shown). Similarly, fluorescence microscopy revealed the appearance of large brightly fluorescent cells in the population after 20 h of incubation with antibiotic in medium without yeast extract, while after 80 h the onset of visible degradation and low-level Rh123 accumulation in these cells was observable (data not shown). Similar results were obtained after incubation of cells in the presence of cerulenin (see reference 17), an inhibitor of lipid biosynthesis (data not shown). Cultivation of cells that had been starved for 5 months and then incubated in the presence of 0.05% yeast extract and chloramphenicol resulted in cell lysis after 20 h of incubation (Fig. 2; see Fig. 5).

The increase in the number of cells in regions B and C (above fluorescence channel 80) during incubation in the presence of chloramphenicol was even more pronounced (Fig. 3). Figure 4a shows that after 47 h of incubation, most cells fluoresced in region B. In contrast to those in region C, not all cells in region B were fully sensitive to CCCP (Fig. 4b), while the percentage of CCCP-sensitive cells increased almost in parallel to the number of cells in region B. The addition of CCCP to the culture after 47 h of incubation resulted in a distribution pattern similar to that before incubation (cf. Fig. 4b and 1c).

The increase in the percentage of Rh123-accumulating cells under these conditions was not accompanied by an increase in either the total count or the viable count of the cells, except with cells incubated in the presence of yeast extract instead of lactate, for which there was a decrease in the total count (Fig. 5). In the last case, the lysis of cells did not affect the viable count (Fig. 5). Similar results were obtained by using plates with lactate minimal medium (data not shown).

Figure 6 shows the results of flow cytometric assays (24) to determine the percentage of cells maintaining a membrane permeability barrier for the nucleic acid stain PO-PRO-3 during the incubation of starved cells in the presence of chloramphenicol in various media. Parallelling the findings with Rh123, this approach revealed a transient restoration of the membrane permeability barrier in 40 to 50% of cells (as the maximum percentage of cells unstainable by PO-PRO-3 during the incubation period) when medium without nutrients or with lactate was used. However, no repair of the permeability barrier was found to occur in medium containing yeast extract, prior to the cell lysis after some 20 h as mentioned above.

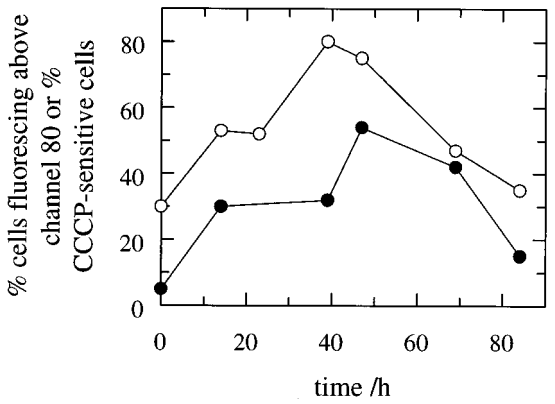

FIG. 3. Changes in the percentage of cells accumulating Rh123 in regions B and C shown in Fig. 1 (i.e., above channel 80) in suspensions of *M. luteus* cells that had been starved for 5 months, during incubation in lactate minimal medium lacking L-lactate but in the presence of chloramphenicol (100 μg/ml). Open circles, no additions; closed circles, treatment with 15 μM CCCP. For other details, see the legend to Fig. 2.

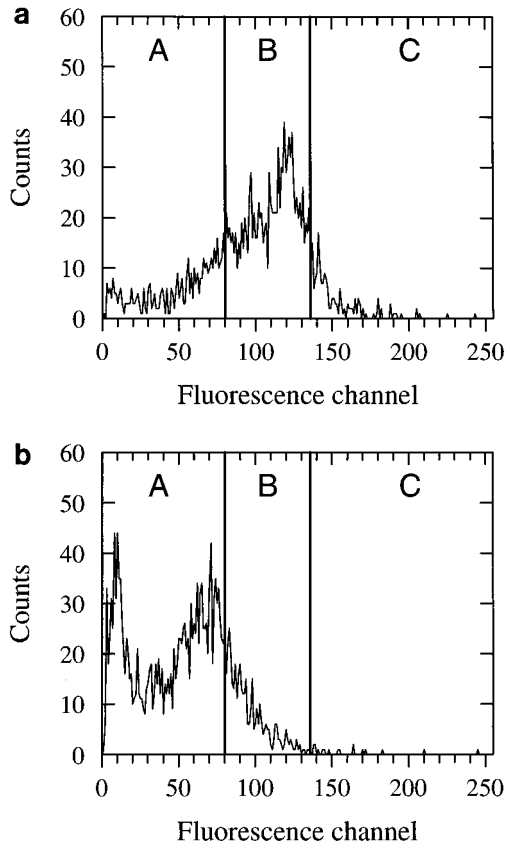

FIG. 4. Flow cytometric distribution of the fluorescence of *M. luteus* cells that had been starved for 5 months and stained with Rh123 after 47 h of incubation in lactate minimal medium lacking L-lactate but supplemented with chloramphenicol (100 μg/ml). Cells were incubated and analyzed as described in the legends to Fig. 1 and 2. (a) Cells before treatment; (b) cells after treatment with CCCP (15 μM).

Cell sorting. We sorted cultures whose fluorescence was of the type displayed in Fig. 1c and Fig. 7 into two populations: (i) cells of which the rhodamine staining was sensitive or partially sensitive to CCCP (regions B and C in Fig. 1) and (ii) cells whose rhodamine-dependent fluorescence was not sensitive to CCCP (region A). After sorting, cells were plated on nutrient agar for viable-count determinations, while the total count of sorted samples was also examined. A control incubation of both starved and fresh cells in flow cytometer sheath fluid for 2 h had no influence on their viability (data not shown). Table 1 shows the results of a typical experiment. Because of the great dilution of samples during sorting (which varies from experiment to experiment), we express all viable-count numbers as those normalized to the total number of cells in a particular region. As would be expected, the great majority of colony-forming cells originated from regions B and C, resulting in an increase of the viability of the population obtained from these regions. However, the number of colony-forming cells in regions B and C and in the whole population after sorting (per 10^6 cells) was unexpectedly and significantly greater (8 to 20 times greater in different experiments) than the number before sorting. Similar results were obtained by using plates with lactate minimal medium (data not shown).

Earlier, we used an MPN assay for the estimation of the number of resuscitable cells in starved *M. luteus* populations (7). The important condition for such an estimation was addition of diluted supernatant from growing bacteria to the media in which the MPN assay was performed. Table 1 demonstrates the resuscitation of cells under such conditions for the whole population before sorting (showing a some-50-fold increase in the viable count when the MPN assay was done in the presence of supernatants with these cultures that had been starved for 5 months). The same effect was observed when cells sorted from regions B and C were subjected to resuscitation, while resuscitation of cells from region A was unsuccessful (Table 1).

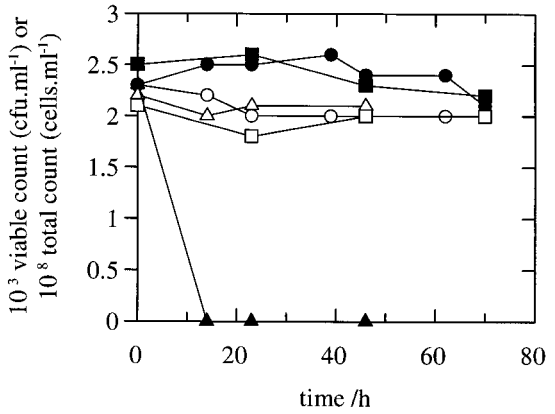

FIG. 5. Changes in the total and viable counts of starved *M. luteus* cells during incubation in different media supplemented with chloramphenicol (100 μg/ml). For details, see the legend to Fig. 2. Closed symbols, total count; open symbols, viable count. The media were lactate minimal medium lacking lactate (circles), the same medium with 0.05% yeast extract (triangles), and the same medium with 0.5% Li lactate (squares). The coefficient of variation for the total count is 6%, and that for the viable count is 5%.

DISCUSSION

In earlier work, we found significant heterogeneity in a variety of *M. luteus* cultures as revealed by flow cytometry of Rh123-stained cells. This was first evidenced after several divisions in a very slowly growing chemostat culture where subpopulations of apparently dormant and viable cells were found (2, 5). After an extended starvation of *M. luteus* in spent medium following growth in batch culture, more than 99% of the

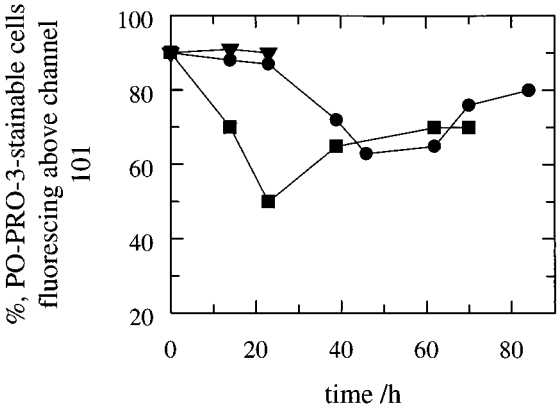

FIG. 6. Changes in the percentage of cells fluorescing above channel 101 in the analytical flow cytometer during incubation of starved *M. luteus* stained with PO-PRO-3 in the presence of chloramphenicol. For incubation conditions, see the legend to Fig. 2. To stain with PO-PRO-3, 0.2 ml of a sample taken from the flask was incubated for 8 to 10 min with 30 μM stain (24). The photomultiplier tube voltage for the fluorescence channel was 600 V.

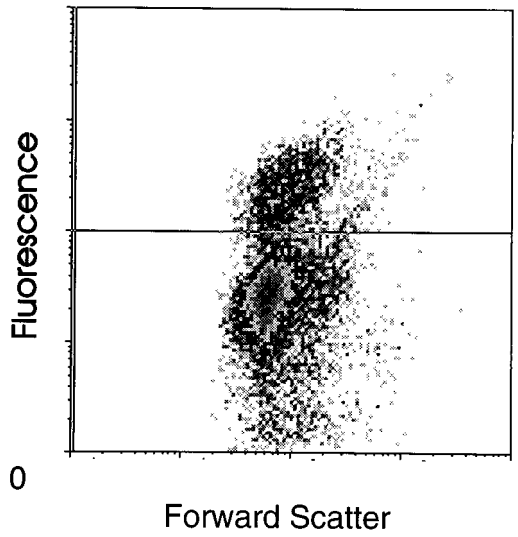

FIG. 7. Distribution of *M. luteus* cells that had been starved for 5 months and stained with Rh123 and subjected to sorting in a flow cytometric cell sorter. For experimental details, see the legend to Fig. 1. The horizontal line shows the sorting region which was used to discriminate between regions A and B plus C. Cells from the two regions were sorted differentially into separate test tubes, examined by the MPN assay and plating, and assessed for their total counts. The scales are logarithmic and cover 4 decades.

cells lost their ability to accumulate Rh123 in a metabolically dependent (CCCP-sensitive) fashion (6). The present study confirmed that cultures starved for 5 months consisted of a very low percentage (less than 2 to 3%) of cells which could actively accumulate Rh123. Despite this, these starved cultures revealed two different types of cells, those with low and those with elevated fluorescence. The fluorescence intensity of cells in population B was comparable to the fluorescence of actively growing *M. luteus* cells, although with the starved cells the fluorescence was almost insensitive to uncoupler, in contrast to that of the actively growing, viable cells. It is reasonable that the increased uncoupler-resistant fluorescence of some cells reflects the changes in surface (membrane) structure (see also reference 14), which could result in increased Rh123 adsorption or fluorescence quantum yield by the cells. Indeed, a characteristic property of starved bacteria (see references 4 and 21) is an increase in their surface hydrophobicity. It is also worth mentioning that the present cells, which had been starved for 5 months, were significantly older than those which we studied in more detail previously (6).

Incubation of cells in the presence of chloramphenicol, but otherwise under the conditions we applied earlier for resuscitation of dormant cells in starved *M. luteus* cultures (6, 24), resulted in a more complex pattern of the distribution of cellular properties, including the appearance of larger, metabolically active cells in region C and a significant increase of the metabolically active cells in region B. This was accompanied by a restoration of the membrane permeability barrier in 40 to 50% of cells.

These experiments, under conditions in which the influence of cryptic growth or growth of initially viable cells can be excluded because of the presence of antibiotics in the medium, clearly show the ability of a significant number of cells (up to 50%) (Fig. 3) in a starved population to recover, at least in terms of metabolic activity, in agreement with our previous conclusion (24). In contrast to what we found in that work, in which the resuscitation was performed in the absence of antibiotics, a significantly greater extent of resuscitation was observed, and it is interesting that this recovery needed neither protein nor lipid synthesis (in that it occurred in minimal medium) and probably reflects the exploitation of endogenous resources by starved cells. Indeed we have found that some enzymes (especially membrane-bound ones) preserve potential activity (on addition of substrate) even after 6 months of starvation of *M. luteus* (14). We have also found that during cell resuscitation there was a much more pronounced restoration of the cell permeability barrier for NADH and restoration of phospholipid composition when chloramphenicol was present in the medium (13). In the presence of yeast extract instead of lactate, protein synthesis evidently takes place during incubation as cells lysed in the presence of chloramphenicol (Fig. 5 and 6). This could reflect an imbalance in cell metabolism in the presence of more easily transformable substrates during the transition from dormancy to an active state, as viable cells did not lyse under similar conditions (data not shown).

The metabolic recovery found in this study is insufficient for the restoration of the ability of such cells to grow on agar plates (Fig. 5), probably, as we reported earlier, because full resuscitation of starved *M. luteus* needs a factor(s) secreted by viable cells into the culture fluid (7, 24), an example of the increasing number of cases of bacterial pheromone production (8).

Several conclusions can be made on the basis of the cell-sorting data. (i) The viable cells in cultures starved for 5 months are indeed concentrated mainly in regions B and C (Fig. 1c). (ii) The viable count of the whole culture, as well as of the population in regions B and C, increased after cell sorting. Sorting serves to separate the different cells and permits the expression of the maximal number of viable cells in the culture. Again, even under such conditions not all of the cells in the population are able to produce colonies (maximally, 3%). (iii) The resuscitation of cells as judged by the MPN assay was successful for cells in regions B and C but not for cells in region A. This constitutes direct evidence that dormant cells are concentrated in regions B and C.

In conclusion, populations of the nonsporulating bacterium *M. luteus* that have been starved for an extended period are

TABLE 1. Total count, viable count, and distribution in viability of cells taken from cultures of *M. luteus* that had been starved for 5 months, before and after flow cytometric cell sorting[a]

Property	Result for cells			
	Before sorting	After sorting		
		Region A	Regions B+C	Regions A+B+C
Total count	10^6	$8 \cdot 10^5$	$2 \cdot 10^5$	10^6
Viable count by plates	740	42	6,200	6,240
Viability by plates (%)	0.07	0.005	3.1	0.62
Viability by MPN (no supernatant added) (%)	0.065	0.0044	1.5	0.3
Viability by MPN (in presence of supernatant) (%)	3	0.004	19.3	3.9

[a] The distribution of the total count among regions A, B, and C was estimated from the flow cytometric distribution pattern (as shown in Fig. 7) normalized to a value for the whole population of 10^6 cells. Viable counts were calculated relative to the total number of cells in a particular region. Lab M medium was used for determining the count of viable bacteria on agar plates (second row). The viable count, and hence viability, by the MPN assay was determined as described in Materials and Methods, except that lactate minimal medium with 0.05% yeast extract was used without (fourth row) or with (fifth row) supernatant taken from a fresh logarithmic-phase *M. luteus* culture. Coefficients of variation for the total and viable counts were 5.6 and 4.4%, respectively.

heterogeneous and consist of viable, dormant, and dead cells, which can influence each other during resuscitation, causing an underestimation of the number of potentially resuscitable cells in the culture. The first two subpopulations could be discriminated and sorted out from the last on the basis of their ability to bind the weakly lipophilic cation dye Rh123. Cell sorting is a powerful method for the analysis of heterogeneity in bacterial systems (10, 18, 19, 22, 23, 26).

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