APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1994, p. 3284–3291
0099-2240/94/$04.00+0

Vol. 60, No. 9

Influence of Viable Cells on the Resuscitation of Dormant Cells in *Micrococcus luteus* Cultures Held in an Extended Stationary Phase: the Population Effect

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Received 9 March 1994/Accepted 30 June 1994

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). In the present work, such cultures were studied by both flow cytometry and conventional microbiological methods and were found to contain various numbers of viable cells. Pretreatment of such cultures with penicillin G, and subsequent dilution, was used to vary this number. When the initial number of colony-forming cells per 30-ml flask was approximately nine (±five) or more, resuscitation of 10 to 40% of the cells, and thus culture growth, was observed. The lag period before the appearance of a population of cells showing significant accumulation of the fluorescent dye rhodamine 123 (i.e., of cells with measurable membrane energization) decreased from 70 to 27 h when the number of viable cells was increased from 30 to 10^5 per flask, while the lag period before an observable increase in the number of colony-forming cells occurred was almost constant (at some 20 h). Provided there were more than nine (±five) initially viable cells per flask, the number of initially viable cells did not affect the final percentage of resuscitable cells in the culture. The lag period could be ascribed in part to the time taken to restore the membrane permeability barrier of starved cells during resuscitation, as revealed by flow cytometric assessment of the uptake of the normally membrane-impermeant fluorescent DNA stain PO-PRO-3 {4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylidene]-1-(3′-trimethylammonium propyl)-pyridinium diiodide}. Although cell populations which contained fewer than nine ±five viable cells per flask failed to grow, 4 to 20% of the cells (of 1.2×10^6) were able to accumulate rhodamine 123 after 80 to 100 h of incubation, showing the ability of a significant number of the cells in the population at least to display "metabolic resuscitation." Resuscitation and cell growth under such conditions were favored by the use of a 1:1 mixture of fresh lactate medium and supernatant from late-logarithmic-phase *M. luteus* cultures as the resuscitation medium. We conclude that the presence of a small fraction of viable cells at the onset of resuscitation facilitates the recovery of the majority of the remaining (dormant) cells. The cell density dependence of the kinetics, or population effect, suggests that this recovery is due to the excretion of some factor(s) which promoted the transition of cells from a state in which they are incapable of growth and division to one in which they are capable of colony formation.

One may define (bacterial) dormancy as "a reversible state of low metabolic activity, in which cells can persist for extended periods without division" (16), and we have recently reported that cells of the nonsporulating gram-positive coccus *Micrococcus luteus* can indeed persist in a dormant state during prolonged incubation in spent growth medium for a lengthy stationary phase following batch growth (16, 18, 19). Two other groups of nonsporulating bacteria may be considered potentially dormant, viz., the so-called viable but nonculturable forms (26, 33) and (at least some of) the ultramicrobacteria (25) found in natural water habitants. For each of these cases (including ours), a characteristic property of these forms is their inability to produce colonies when plated directly onto solid agar media (for a review, see reference 16), and it has been suggested that dormant cells can be converted to normal, colony-forming bacteria following a special resuscitation procedure involving precultivation in liquid media (1, 16, 18, 23).

However, given the imperfect precision of total-cell-count measurements, such studies are always potentially subject to the criticism that an increase in the viable-cell count at a nominally constant total cell count may be due not to resuscitation but to the possible multiplication during the resuscitation period of a small number of cells which were viable from the beginning (24, 32, 40). Nonetheless, using flow cytometry with appropriate fluorescent probes and suitable conditions of incubation, we found that these difficulties can be overcome so that neither the rate, the extent, nor the physiological character of the resuscitation observed could be accounted for in terms of the ongrowth of initially viable cells. Under our conditions, at least 50% of *M. luteus* cells in 3-month-old populations could be resuscitated, and hence those cells were dormant (18). However, older cultures in which the initial number of colony-forming cells was statistically indistinguishable from zero demonstrated only 'metabolic resuscitation" (the appearance of membrane energization) and not growth (18).

By exploiting the most-probable-number approach and by resuscitating cells in media which contained (statistically) no initially viable cells but which were fortified with sterile (filtered) supernatant from the late logarithmic phase of batch growth, we showed that growing cultures could produce a resuscitation-promoting factor which could promote the resuscitation of dormant cells (20) in the MPN assay. While the culturable cells in the extended-stationary-phase populations

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did not contribute to the increase in viable-cell counts observed during resuscitation by dividing, the question of whether they may have promoted the resuscitation of the other, dormant cells by excreting such a factor or factors arose. Indeed, a correlation of the initial cell density with the ability of starved copiotrophic bacteria to survive (as judged by viable-cell-count methods), known as the population effect, has long been established (10, 28, 29, 38, 39). We therefore studied the resuscitation of starved and mainly dormant cultures in which the concentrations of viable cells present prior to the resuscitation were varied. We found that the lag before the cells observably began to resuscitate depended significantly on the initial size of the population of viable cells, with kinetics consistent with the view that such initially viable cells did indeed secrete a dormancy-breaking or resuscitation-promoting factor. In addition, we showed that the lag periods could be ascribed at least in part to the time taken to restore the membrane permeability barrier of starved cells during resuscitation, as revealed by flow cytometric assessment of the uptake of the normally membrane-impermeant fluorescent DNA stain PO-PRO-3 {4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylidene]-1-(3′-trimethyl-ammonium propyl)-pyridinium diiodide}.

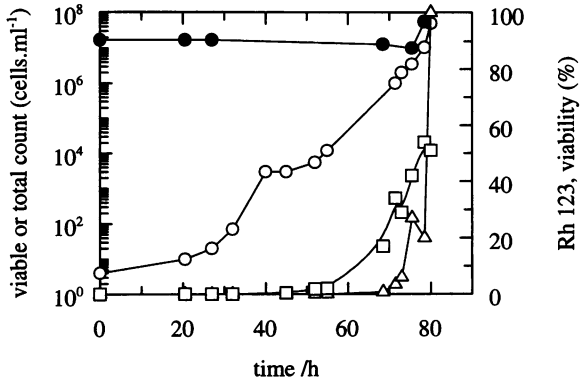

FIG. 1. Changes in viable-cell and total cell counts, percent viability, and percentage of cells accumulating Rh123 during resuscitation of starved *M. luteus* cells. Cells were starved for a total of 4.5 months, incubated with penicillin G, washed, and resuscitated as described in Materials and Methods. The concentration of cells (total count) at the onset of resuscitation was 1.7×10^7 ml^{-1}. Symbols: open circles, viable-cell count per milliliter (by agar plating); solid circles, total count per milliliter; open triangles, percent viability; open squares, percentage of cells fluorescing in a channel number above 65.

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 (30-ml medium volume) in a lactate minimal medium containing L-lactate, exactly as described previously (17).

Starvation and resuscitation. When the culture had reached stationary phase (some 4.10^9 cells ml^{-1}), agitation was continued at 30°C for up to 3 months. Cultures were then held aerobically at room temperature without agitation for various periods up to a further 3 months. Before resuscitation, cells were diluted 10-fold into lactate minimal medium containing 0.05% (wt/vol) yeast extract and 0.1 to 0.5 μg of penicillin G per ml. The cell cultures were incubated aerobically at 30°C with agitation for 20 to 24 h. Then cells were washed twice with minimal medium minus lactate (although there was no difference if lactate was included) and inoculated into fresh lactate minimal medium containing 0.05% yeast extract. Given that penicillin does not kill all the cells under these conditions (18), a range of dilutions (from 100- to 100,000-fold) of the penicillin-pretreated, washed cell suspension was employed to produce the inocula which were used in these resuscitation media. Also, because different experiments did not result in the same number of viable cells following the incubation with penicillin, the ratio of viable cells/total cells also varied slightly, normally being in the range of 3×10^{-8}:1 to 8×10^{-8}:1. The resuscitation was carried out with agitation at 30°C.

For the preparation of sterile supernatants, fresh cultures of *M. luteus* were grown aerobically at 30°C in lactate minimal medium until late logarithmic phase and centrifuged, and the supernatants were passed through a 0.22-μm-pore-size filter (Gelman or Millipore).

Flow cytometry. The flow cytometric analysis of the distribution of cells in populations in experiments with rhodamine 123 (Rh123) uptake was performed exactly as described previously (17, 21) with a Skatron Argus 100 instrument. To stain cells with PO-PRO-3 (supplied as a 1 mM solution in water/dimethyl sulfoxide [4:1]), cells were incubated in lactate minimal medium with (normally) 30 μM PO-PRO-3 at room temperature for 5 min. To detect fluorescent cells, a G-1 fluorescent filter block (excitation, 530 to 550 nm; band stop, 560 nm; emission, >580 nm) was used. The photomultiplier tube voltages were 400 V for the forward light scattering channel and approximately 600 V for the fluorescence channel. To ensure probe equilibration with the target, at least three passages of the sample through the instrument were done for each measurement. All samples were carefully dispersed before flow cytometry by repeated extrusion through a 0.4-mm-diameter hypodermic needle to eliminate clumps of organisms.

CFU and total cell counts. The number of viable cells (CFU) was estimated by plating cells on agar plates containing Nutrient Broth E (Lab M) and 1.5% (wt/vol) purified agar (Lab M no. 1) as previously described (17). Total cell counts of unstained cells were performed with a phase-contrast microscope and a modified Neubauer counting chamber. In experiments with low cell concentrations ($<5.10^7$ ml^{-1}), total cell counts were performed in the flow cytometer with a diluted sample of *M. luteus* cells of known cell concentration as a reference. To obtain total cell counts during the resuscitation experiments, samples were collected and immediately frozen at −20°C; the total count was then performed by flow cytometry for all the samples seriatim on the same day.

Chemicals. Nutrient Broth E, yeast extract, and agar were obtained from Lab M, while L-lactate (Li salt), penicillin G, and Rh123 were obtained from Sigma. PO-PRO-3 (11) was obtained from Molecular Probes, Eugene, Oreg. Other chemicals were of analytical grade.

RESULTS

Viability and Rh123 accumulation during resuscitation of *M. luteus* cells and influence of initial concentration of viable cells on resuscitation. For resuscitation of starved *M. luteus* cells, we used the same general procedure that we described previously (18). However, for the present experiments, we wished to decrease significantly the concentration of initially colony-forming (viable) cells and therefore diluted the culture after penicillin G pretreatment by 100- to 100,000-fold. Then we examined the behavior of cell cultures with different initial concentrations of viable cells during resuscitation. Figure 1 shows typical results for a resuscitation experiment in which

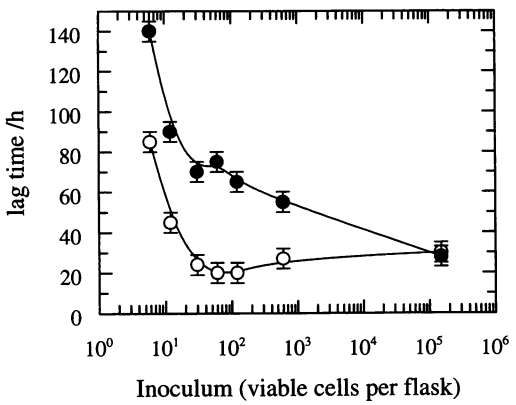

FIG. 2. Effect of the number of colony-forming, viable cells present at the onset of resuscitation on the lag periods during resuscitation of starved *M. luteus* cells before the increase in colony-forming-cell numbers and in the appearance of the ability of at least 0.5% of the cells to accumulate Rh123 to a flow cytometer fluorescence channel above 65. Resuscitation was performed under conditions similar to those described in the legend to Fig. 1, save that the inoculum size was varied as indicated. Symbols: open circles, viable cells; solid circles, Rh123-accumulating cells.

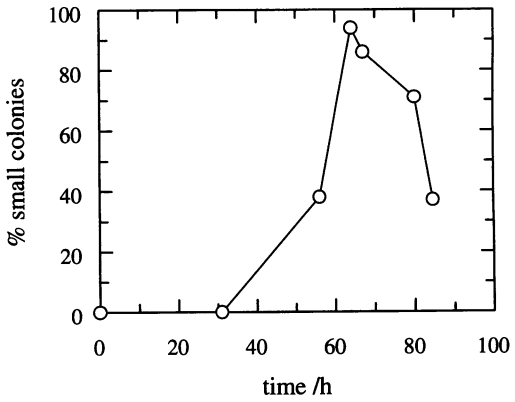

FIG. 3. Changes in the percentage of small, late colonies which appeared on agar plates during the resuscitation of starved *M. luteus* cells. The conditions of the experiment are described in the legend to Fig. 1.

the initial concentration of viable cells was 4/ml (120 per flask). There are discernible lag periods both between the onset of incubation and the beginning of the increase in viable-cell count (20 h, defined as the time point at which the viable-cell count has increased by at least 20%) and between the onset of incubation and the beginning of a noticeable increase in the percentage of Rh123-accumulating cells (55 h, defined as the time point at which >0.5% of the objects observed in the flow cytometer accumulate Rh123 to a level giving a fluorescence signal above channel 65). Both lag periods so defined depended on the initial concentration of viable cells (Fig. 2). However, while the lag time for the appearance of rhodamine-accumulating cells continued to decrease as the viable-cell concentrations was increased from 30 to 10^5 per flask, the lag time before the appearance of viable cells remained essentially constant. For all the initial viable-cell concentrations tested (except the highest), there was a plateau region in the viable-cell count following its initial increase (this occurred at 40 to 55 h in the experiment whose results are shown in Fig. 1), as well as a difference in viability as measured by plating and as assessed from rhodamine accumulation by flow cytometry between 60 and 75 h (Fig. 1).

The colonies which appeared on plates from samples taken after 20 h of resuscitation included both early, large colonies (visible after a 4-day incubation of the plates) and late, small colonies (with a diameter of <1 mm and visible after a further 4 to 10 days of incubation). It is worth noting that the ratio of the different colonies which could be observed on the same plate was a function of the time of resuscitation. Figure 3 shows that the maximum percentage of small colonies was registered after 50 to 70 h of incubation in the experiment for which the results are shown in Fig. 1.

The decrease of the inoculum size to below 30 viable cells per flask led to a significant increase in the lag periods (Fig. 2), while a further decrease of the concentration of initially viable cells in the inoculum below a threshold resulted in the absence of colonies on plates from samples taken from cultures which were incubated for up to 180 h. This threshold number was six viable cells per flask in the experiment for which the results are shown in Fig. 1, statistically nine ± five (mean ± standard deviation) in 12 experiments. However, under these conditions, flow cytometry revealed a transient increase in the percentage of cells able to accumulate Rh123 (Fig. 4 and 5). The maximum percentage of such metabolically active cells (80 to 100 h of incubation) varied from 4 to 20% in five different experiments.

In order to better promote the resuscitation of cells under such conditions, we used as a medium for resuscitation a 1:1 mixture of lactate minimal medium containing 0.05% (wt/vol) yeast extract and supernatant obtained after centrifugation of late-logarithmic-phase *M. luteus* cells. Under such conditions, a gradual increase of both rhodamine-accumulating cells (lag period of ca. 40 h) and colony-forming cells (lag period of 20 to 25 h) in the culture (Fig. 6) was again found.

Membrane permeability barrier in individual cells during resuscitation. To clarify the possible reasons for the existence of lag phases during cell resuscitation, as observed, for instance, in the results shown in Fig. 1 and 2, we studied the state

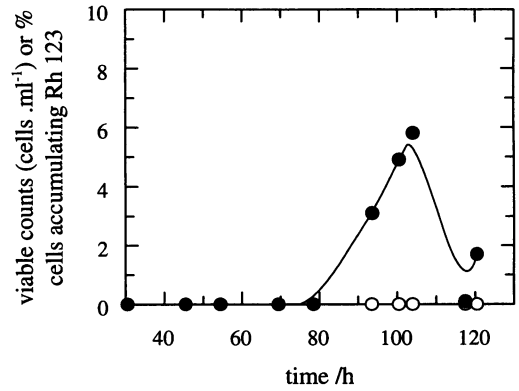

FIG. 4. Changes in the viable-cell count and in the percentage of cells able to accumulate Rh123 during resuscitation of starved *M. luteus* cells. The concentration of colony-forming cells at the onset of resuscitation was 0.2 cells per ml (6 cells per flask). The initial total count was 7.10^6 ml^{-1}. For further experimental details, see the legend to Fig. 1. Symbols: open circles, viable-cell count; solid circles, percentage of cells accumulating rhodamine to a channel number greater than 65.

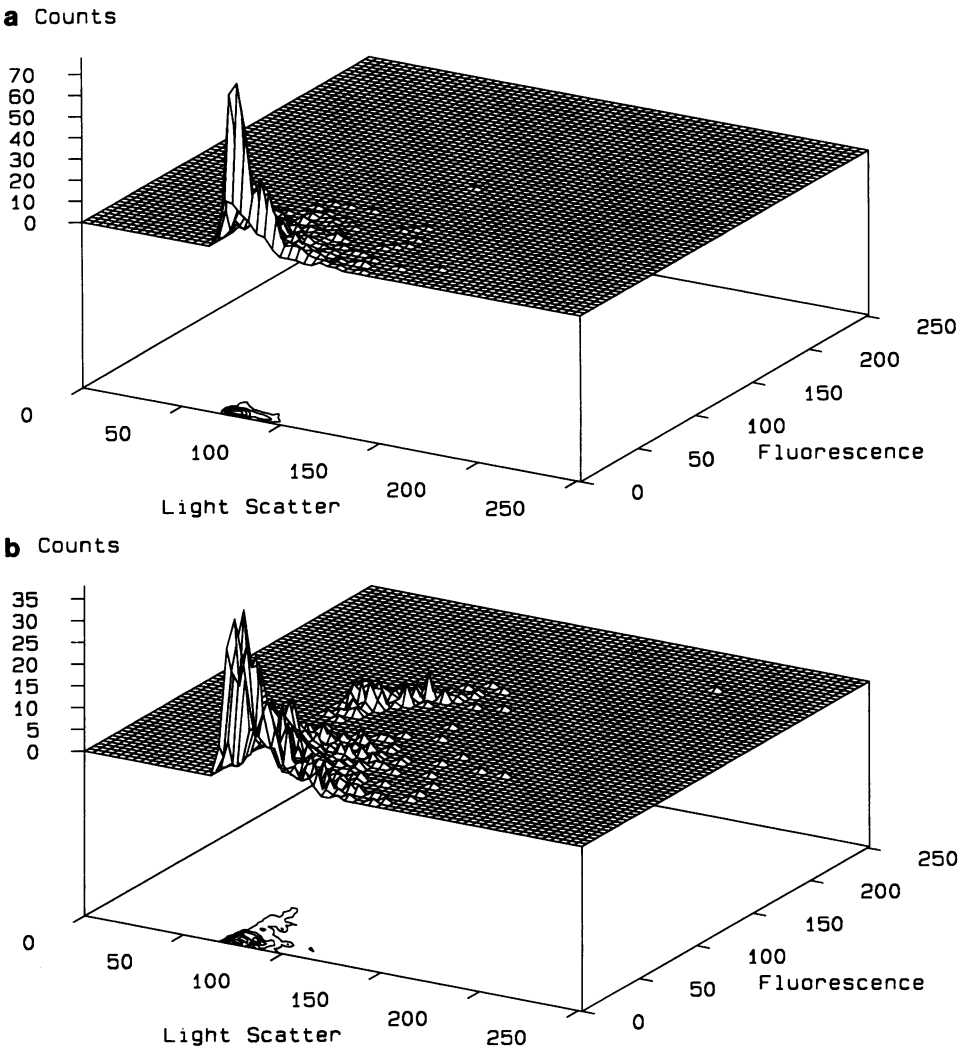

FIG. 5. Changes in the ability of starved *M. luteus* cells to accumulate Rh123 during resuscitation. Cells (4.5 months old) were resuscitated and assessed by flow cytometry for the ability to accumulate Rh123, as described in Materials and Methods. The total cell number and number of viable cells at the onset of resuscitation were 7.4×10^6 ml^{-1} and 0.2 ml^{-1} (6 per flask), respectively. (a) Onset of resuscitation; (b) after 100 h of incubation. Data were displayed with the SKATGRAF program (6).

of the permeability barrier represented by the cytoplasmic membrane in starved *M. luteus* cells before and during resuscitation. To this end, we used the recently developed probe PO-PRO-3, which fluoresces significantly only after binding to DNA (i.e., inside the cell). Moreover, this probe is considered membrane impermeant (11). Indeed, the use of PO-PRO-3 at concentrations of up to 80 μM did not result in the appearance of fluorescent staining in freshly grown cells of *M. luteus* as judged by flow cytometry (Fig. 7), while the concurrent addition of octanol (which is an excellent membrane-permeabilizing agent) (5, 37) included significant fluorescence in almost 80% of the particles in the population (Fig. 7). In contrast to cells from freshly grown cultures, *M. luteus* cells starved for 5 to 6 months were well stained by PO-PRO-3, indicating the existence of a damaged permeability barrier in at least 80% of the population (Fig. 7 and 8). We further found that the distribution of cellular properties as judged by flow cytometry was not changed upon increase of the time of incubation with the probe or upon variation in the cell concentration (data not shown). In further experiments, we used PO-PRO-3 under standard conditions to monitor the state of the permeability barrier during cell resuscitation (see Materials and Methods). Figure 9 shows that starved cells were still leaky after 22 h of incubation with penicillin G (which represents the zero time of resuscitation); however, after the first 20 to 40 h of incubation in resuscitation medium, the percentage of stainable cells decreased to <20 to 40% (in five different experiments) and remained at this level during further incubation.

DISCUSSION

In the present resuscitation experiments, we used an inoculum containing a much lower concentration of starved bacteria after penicillin G pretreatment than those described previously (18). Notwithstanding this difference, the shapes of the curves shown in Fig. 1 were similar to those previously obtained with a higher initial cell concentration (18), and the data again showed a measurable difference in percent viability as judged

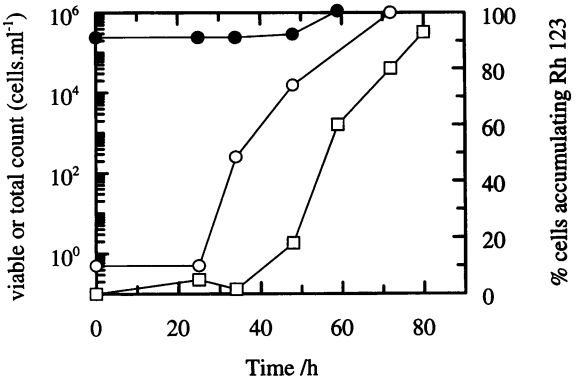

FIG. 6. Changes in viable-cell and total cell counts and in the percentage of cells accumulating Rh123 during the resuscitation of starved *M. luteus* cultures containing only a very small number of viable cells. The conditions for resuscitation were similar to those described in the legend to Fig. 1, save that the medium for resuscitation was a 1:1 mixture of lactate minimal medium containing 0.05% yeast extract and supernatant from a late-logarithmic-phase *M. luteus* culture and that the concentration of colony-forming cells at the onset of resuscitation was 0.46 cells ml^{-1} (a number insufficient for this culture to resuscitate without the supernatant) (data not shown). Symbols: open circles, viable-cell count; solid circles, total cell count; open squares, percentage of rhodamine-accumulating cells.

from plate counts and from rhodamine accumulation detected by flow cytometry towards the end of resuscitation (when the percentage of rhodamine-accumulating cells was sufficient to be measurable with some precision). This difference shows that membrane energization (reflecting activated metabolism) preceded the transformation of cells from a state in which they are nongrowing to one in which they can form colonies.

The broadly inverse dependence of the duration of the lag phase on the inoculum size (viable bacteria) found in this study (Fig. 2) accords in part with the well-known dependence of the lag phase of conventional bacterial cultures on inoculum size and is best explained in terms of the density-dependent excretion of one or more chemicals which either activate resuscitation or remove a dormancy-maintaining factor. However, while the lag phase between the onset of incubation and the beginning of the increase in colony-forming-cell numbers was almost independent of the initial concentration of viable cells (which varied between 30 and 10^5 cells $flask^{-1}$), the lag period for the appearance of a cell fraction accumulating Rh123 was decreased from 70 to 20 h. Clearly, the thresholds for the sensitivity of cell counting by flow cytometry and by plating are different (the appearance of 1,000 newly viable cells in a culture containing a total of 10^7 cells ml^{-1} is not seen by flow cytometry). However, if the appearance of Rh123-accumulating cells after 60 h of incubation were to be caused by the multiplication of initially viable cells until they achieved some detectable level, the lag period as judged by flow cytometry should also be expected to be constant over the same range of inoculum sizes (given an independence of the cell generation time during growth from the initial concentration of viable cells). It is worth noting that the sensitivity of flow cytometry is determined by the ratio of cells with different properties and not by their absolute concentrations. Finally, the existence of the plateau in the middle of the resuscitation also argues against the appearance of viable cells being due to their reproduction. Thus, these experiments provide further evidence that the appearance of Rh123-accumulating cells is due to resuscitation and not to ongrowth and support our earlier conclusions concerning the resuscitation of dormant *M. luteus* cells after cultivation in liquid medium (18).

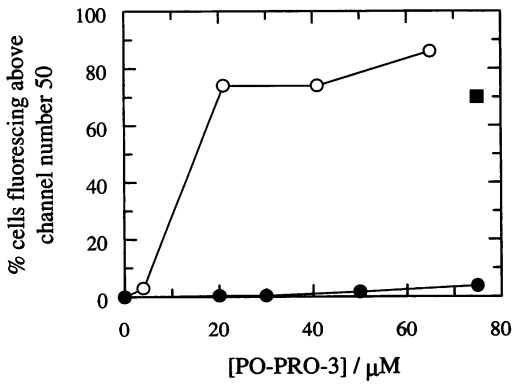

FIG. 7. Dependence on PO-PRO-3 concentration of the effectiveness of staining of *M. luteus* cells. A total of 5.10^8 cells ml^{-1} in lactate minimal medium without lactate were stained with PO-PRO-3 for 5 min and then analyzed by flow cytometry as described in Materials and Methods. The photomultiplier tube voltage for the fluorescence channel was 600 V. There was no signal above channel number 50 in the fluorescence window of unstained cells, nor was there any dependence of cell staining on cell concentration or on any further increase in the incubation time. When present, octanol was added at 6 μl/ml of a 1:10 (vol/vol) mixture with ethanol 20 min prior to analysis. Symbols: open circles, cells starved for 5 months; solid circles, freshly grown cells harvested at early stationary phase; solid square, freshly grown cells harvested at early stationary phase and treated with octanol.

It is probable that towards the end of the resuscitation period, not all cells capable of resuscitating have done so before some of the resuscitated cells begin to reproduce, i.e., at this stage, resuscitation and the multiplication of now-viable cells begin to occur simultaneously; this would lead to uncertainty in the calculation of the final concentration of resuscitable cells from the viable-cell count. Indeed, by this period (ca. 80 h in the experiment for which the results are shown in Fig. 1), the apparent "generation time" is less than 40 min, a rate much higher than any that has been found for the moderately slowly growing *M. luteus* under these conditions. The fast rise in the counts of viable and total cells at the end of the incubation can be explained only by an overlap of growth and resuscitation, which in turn results in an underestimation of resuscitable cells from the viable-cell counts as judged by plating. The existence of heterogeneity in the size and number of colonies which appear on agar plates in samples taken after 40 h of incubation also supports this suggestion. It may well be that late, small colonies reflect the appearance of cells in liquid medium that are able to resuscitate further directly on agar plates, since the decrease in the percentage of such cells coincides in time with the onset of the intensive multiplication of cells (at more than 80 h).

The loss of cell viability is well known generally to be associated with a loss of the membrane permeability barrier, and many so-called viable strains of course rely on this principle to distinguish biomass from necromass (for example, see reference 9). It was therefore of interest to establish whether dormant cells possessed intact or leaky membranes. Indeed, we found that the lag phase before colony-forming cells increased (20 h) could indeed be correlated with a restoration of the membrane permeability barrier. The use of the normally membrane-impermeant DNA stain PO-PRO-3 and flow cytometry revealed that almost 80% of the cells in the

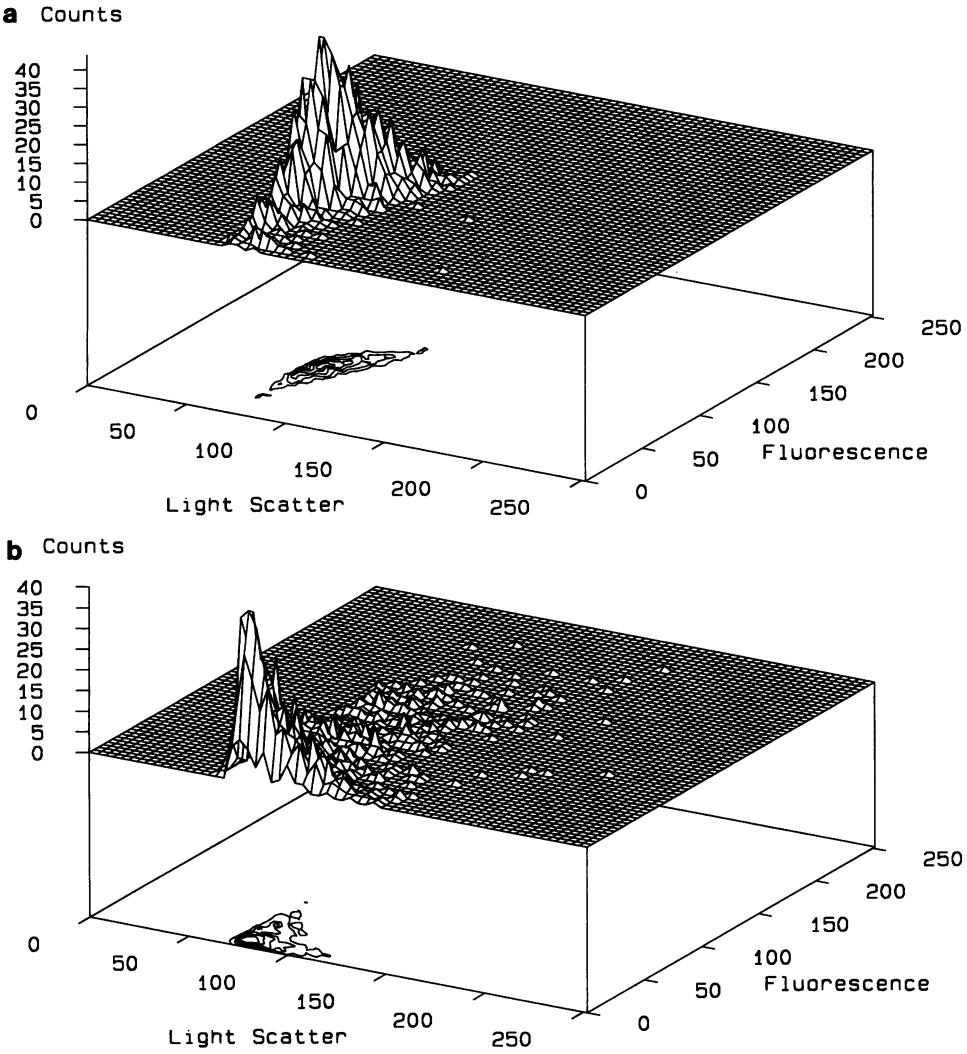

FIG. 8. Changes in the ability of starved *M. luteus* cells to stain with PO-PRO-3 during resuscitation. (a) At the beginning of the resuscitation; (b) after 65.5 h of resuscitation. Flow cytometry was performed with photomultiplier tube voltages for the light-scattering and fluorescence channels of 400 and 600 V, respectively. For experimental details, see Materials and Methods and the legend to Fig. 7.

population had a damaged barrier before resuscitation (Fig. 8 and 9). This cell number was likely underestimated (i.e., the real percentage of damaged cells is close to 100%), since octanol administration did not increase the percentage of "cells" stainable by PO-PRO-3 to above 80%. In this case, this margin probably reflected the heterogeneity of the cells in the culture with respect to their DNA content (18) (or, more accurately, the heterogeneity of the particles capable of scattering light and on which the flow cytometer was gated). Evidently, a cell which is to be resuscitated must repair its membrane permeability barrier first. In this regard, flow cytometry showed that in the first 20 to 30 h of resuscitation (well before Rh123 accumulation by the cells was observable), the fraction of cells which had repaired their permeability barrier increased to up to 50 to 70%. (A similar restoration of the barrier properties of the membrane has been found in bacteria cultivated in fresh medium after freeze-thawing and other stresses [13, 31].) While the repair and possession of an intact membrane are presumably conditions that are necessary for a cell to resuscitate and subsequently to grow under these conditions (8), they are not sufficient ones, in that the maximum percentage of resuscitable cells in the cultures used in this study was estimated (from nine experiments) to be 10 to 20% (from viable-cell counts) or 20 to 40% (from rhodamine accumulation by flow cytometry). These estimations were carried out according to the method elaborated previously (18) and were taken from the viability and Rh123 accumulation curves (Fig. 1), which were based on the maximum viability as determined by plate counting or by the percentage of Rh123-accumulating cells under conditions of a constant total count of cells during the resuscitation (before 79 h for Fig. 1).

Summarizing, we suggest that the following sequence of events occurs during the resuscitation of cultures under conditions similar to those used for the experiment for which the results are shown in Fig. 1: (i) 0 to 20 h (lag phase), recovery of the cell membrane permeability barrier; (ii) 20 to 40 h, increase in the concentration of metabolically active cells able to produce colonies during further prolonged incubation and resuscitation (10 to 15 days) on an agar surface but unable to multiply rapidly in liquid medium in this time period (note the

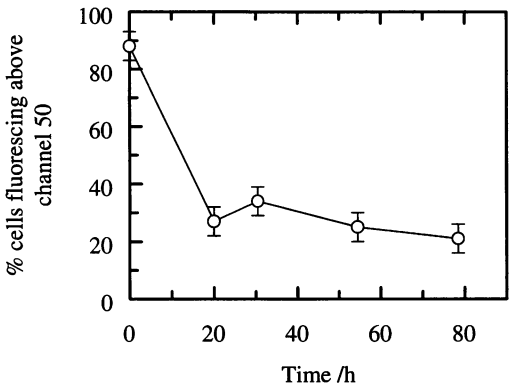

FIG. 9. Changes in the percentage of cells fluorescing during resuscitation of *M. luteus* cells when the cells were stained with PO-PRO-3. For resuscitation conditions, see the legend to Fig. 1. The cells were starved for a total of 4.5 months, incubated with penicillin G for 22 h, washed, and resuscitated as described in Materials and Methods. To stain the cells with PO-PRO-3, a 0.2-ml sample was taken from the flask and incubated for 5 min with 30 μM stain. Flow cytometry was performed as described in the legend to Fig. 7. Time zero represents the beginning of the resuscitation period.

existence of a plateau in the viable-cell counts within 40 to 55 h); (iii) 55 to 78 h, resuscitation of a significant number of cells in the population and an increase in the proportion of cells accumulating Rh123 and able to reduce 5-cyano-2,3-ditolyl tetrazolium chloride (a marker of cell respiration [19]); and (iv) 78 h and beyond, further resuscitation accompanied by cell multiplication, visible culture growth, and an increase in total cell counts.

An important result of this study is thus the demonstration that the presence of viable cells in a population before resuscitation is a requirement for the recovery of dormant cells. The number of viable (colony-forming) cells critical for resuscitation to occur was found to be nine ± five cells per 30 ml. There was no culture growth if the initial concentration of viable cells was below this level. Nevertheless, flow cytometry revealed the transient appearance after 100 h of incubation of a significant number of cells (5 to 20%) with the ability to accumulate rhodamine. Thus, under these conditions, starved cells were able to recover at least partially in terms of their metabolic activity but unable to go on to divide despite the restoration of their permeability barrier as revealed by a decrease of the cell fraction stained with PO-PRO-3 (data not shown). Evidently, metabolically resuscitated cells needed some factor(s) excreted from viable cells for the further maintenance of their metabolic activity and for the promotion of cell multiplication. The presence of such a factor(s) in cell cultures also clearly caused a shortening of the lag phase before Rh123 accumulation during cell resuscitation, and we have recently found that supernatants taken from late-log-phase batch cultures of *M. luteus* greatly stimulated the resuscitation of starved cells at high dilutions, as judged by the most-probable-number method (20). Usage of this approach in the present study indeed resulted in the successful resuscitation of cultures with a concentration of viable cells less than the critical number and in a substantial shortening of the lag prior to observable Rh123 accumulation (Fig. 6).

We believe that these findings, taken together, may help to explain some of the difficulties and controversial published results obtained in experiments on resuscitation in which the initial numbers of viable cells in a population which may actually promote the resuscitation of dormant cells were different (1, 4, 23, 24, 32, 40). The existence of a population effect for the survival of starved bacterial cells and of cells suffering other stresses has long been established (10, 28, 29, 38, 39), while cognate phenomena of dormancy and of the social control of cell survival are now being detailed even for higher eukaryotic cells (for example, see references 30, 35, 36, 41, 43, and 44). Finally, an increasing number of cases in which small diffusible signal molecules of a known structure can mediate a variety of cell density-dependent biochemical changes in prokaryotes are now being recognized (2, 3, 7, 12, 14, 15, 22, 27, 34, 42, 45); it is clear that further progress in the characterization of the properties of bacterial dormancy-breaking factors must include their chemical identification.

ACKNOWLEDGMENTS

We are indebted to the Biotechnology Directorate of the Science and Engineering Research Council, United Kingdom, and the Royal Society, under the terms of the Royal Society/Russian Academy of Sciences Joint Project scheme, for financial support of this work.

We thank J. Gareth Morris and John R. Postgate for many useful discussions and Galina V. Mukamolova for the preparation of starved cultures.

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