

## Dormancy in Stationary-Phase Cultures of *Micrococcus luteus*: Flow Cytometric Analysis of Starvation and Resuscitation

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Cultures of the copiotrophic bacterium *Micrococcus luteus* were stored in spent growth medium for an extended period of time following batch culture. After an initial decrease, the total cell counts remained constant at approximately 60 to 70% of the counts at the beginning of storage. The level of viability, as judged by plate counts, decreased to less than 0.05%, while respiration and the ability to accumulate the lipophilic cation rhodamine 123 decreased to undetectable levels. However, using penicillin pretreatment (to remove viable cells) and flow cytometry and by monitoring both the total and viable counts, we found that at least 50% of the cells in populations of 75-day-old cultures were not dead but were dormant. Resuscitation in liquid medium was accompanied by the appearance of a population of larger cells, which could accumulate rhodamine 123 and reduce the dye 5-cyano-2,3-ditolyl tetrazolium chloride to a fluorescent formazan, while a similar fraction of the population was converted to colony-forming, viable cells. We surmise that dormancy may be far more common than death in starving microbial cultures.

There have recently been extensive discussions concerning the physiological states of vegetative, nonsporulating bacteria during progressive nutrient starvation (29-31, 46, 47, 53), particularly the physiological states of cells in natural oligotrophic environments, such as marine or river habitats. Such bacteria, which are mainly gram negative, often form ultramicrobacteria (10, 34, 35) or so-called viable but nonculturable forms (46), which may sometimes be considered dormant forms (17, 20, 38, 52). A characteristic property of these forms is their inability to produce colonies on solid agar media. Dormant cells are distinguished from dead cells by virtue of the reversibility of the dormant state, resulting in cells that produce colonies on solid media. However, in only a limited number of publications has it been shown that these forms can start to multiply on solid media after the application of a suitable resuscitation procedure (in particular, precultivation in nutrient-containing liquid media) (3, 27, 37, 54).

However, particularly in studies of *Aeromonas salmonicida*, it has been stressed (33, 45) that a limited number of genuinely viable cells in a starved population could have been responsible for the growth that occurred at the end of resuscitations of this type. The same criticism can be applied to many other experiments in which resuscitation procedures were used (but in which total cell counts were not compared with viable cell counts). The main difficulty in such experiments is the fact that bacterial populations even under laboratory conditions are much more heterogeneous than is normally assumed (22, 28). To effect progress, we need to monitor the properties of individual cells rather than the properties macroscopically observable in the whole population (21, 22).

It is reasonable to assume that the phenomenon of dormancy in nonsporulating bacteria is not limited to aquatic

bacteria, which seem to be specially adapted to oligotrophic conditions (11, 41). Indeed, there is evidence of formation of dormant states in normally copiotrophic bacteria after prolonged cultivation in chemostats at very low dilution rates (13, 40). Recently, using flow cytometry and the fluorescent dye rhodamine 123, we have found, as part of an extensive bioenergetic study of this organism, that approximately 40% of *Micrococcus luteus* cells in a chemostat-grown population at a low dilution rate persist as dormant forms, which can be resuscitated to normal bacteria after cultivation under appropriate conditions (18).

Another possible source of dormant bacteria as defined above is cells in the so-called "death phase" following entry into the stationary phase in batch cultures, since the normal "benchmark" criterion of "death" under these circumstances is loss of the ability to form a colony on an agar plate (12, 43). It is well known that the transition from exponential growth to the stationary phase results in numerous changes in cell morphology and in physical and biochemical properties, including RNA degradation, a decrease in the ATP content, expression of some novel "starvation" genes, elevated resistance of stationary cells to different kinds of stress, etc. (16, 20, 24, 25, 29-31, 47, 53). It is at least plausible that cells in this phase prepare to survive nutrient-poor conditions by forming dormant cells. Unfortunately, our knowledge of the behavior of cells in the nominal death phase is very limited, and the question of their physiological status has been little studied since the review of Postgate (42) in 1967. Very recently, Kolter (24) described the expression of special genes and the synthesis of new proteins in "old" cultures of *Escherichia coli*; one of these proteins had a stabilizing effect on DNA in vitro.

The purposes of this study were to establish suitable starvation conditions in old cultures of the copiotrophic bacterium *M. luteus* which resulted in the appearance of large numbers of dormant cells and to develop conditions which were suitable for the resuscitation of such forms into normal bacteria yet were not subject to the crucial criticism

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described above. Using antibiotic pretreatment and flow cytometry and by monitoring both total and viable counts, we found that at least 50% of the cells in populations in 75-day-old cultures were definitely dormant, since they did not initially form colonies, but following resuscitation could be converted to colony-forming cells.

## MATERIALS AND METHODS

**Organism and media.** *M. luteus* Fleming 2665 was maintained on nutrient agar (Difco). Bacteria were grown aerobically at 30°C in shake flasks in lactate minimal medium containing 0.5% (wt/vol) L-lactate and other components described previously (18). The results of growth yield studies suggested that exhaustion of lactate triggered transition to the stationary phase. In some experiments this medium was supplemented with 0.05% yeast extract.

**Starvation and resuscitation.** After the cultures had reached the stationary phase, agitation was continued at 30°C for 12 to 14 days. To avoid evaporation, cultures were then kept at room temperature without agitation in closed bottles. Periodically, samples were withdrawn to check the optical density, the respiration rate, the total and viable counts, and the flow cytometric behavior. Samples for DNA and protein staining were diluted with ethanol to a final ethanol concentration of 77% and kept in a freezer (−20°C) until they were used. For resuscitation, 8 to 10 ml of culture was centrifuged and washed twice with sterile growth medium lacking lactate. The cells were then inoculated to a cell density of approximately  $2 \times 10^8$  to  $3 \times 10^8$  cells per ml into fresh medium containing 0.5% (wt/vol) L-lactate and 15 µg of penicillin G (Sigma) per ml; a sterile solution of penicillin G was prepared by using 0.2-µm-pore-size sterile acrodisc filters (Gelman Sciences). After incubation with penicillin, typically for 10 h, the cells were washed three times with growth medium without lactate and then inoculated into fresh medium (with lactate and with or without yeast extract) to the same cell density.

**Flow cytometry.** In a flow cytometer, individual particles pass through an illumination zone, and appropriate detectors, gated electronically, measure the magnitude of a pulse representing the extent of light scattered or, when optical filters are used, the magnitude of a particle's fluorescence. The magnitudes of the pulses are sorted electronically into "bins" or "channels," permitting the display of histograms consisting of number of cells with a certain property versus channel number. Flow cytometric experiments were carried out as described previously (5, 6, 18, 22) by using a Skatron Argus 100 instrument (Skatron, Ltd., Newmarket, Suffolk, United Kingdom). This instrument (1, 7, 49) contains low-angle (<15°) and large-angle light-scattering detectors plus a variety of fluorescence detectors that can be selected with appropriate optical filters. The sensitivity is determined by varying the voltages on the photomultiplier tube (PMT) detectors; the voltages used in this study are indicated in the legends to the figures. Low-angle light-scattering data were converted to apparent cell diameters by calibrating the instrument with latex beads having known diameters (4, 5); since cells and latex beads scatter light differently, this method uniformly underestimated the diameter of unfixed cells by approximately 26% (5). To detect rhodamine 123 and fluorescein isothiocyanate fluorescence, a fluorescent filter block with the following characteristics was used: excitation at 470 to 495 nm, band stop at 510 nm, and emission at 520 to 550 nm. For DNA detection the relevant parameters were as follows: excitation at 395 to 440 nm, band stop at 460 nm,

and emission at ≥470 nm. Where indicated below, samples were stained with rhodamine 123 (final concentration, 0.3 µM) in 50 mM potassium phosphate buffer (pH 7.4) for 45 to 60 min at room temperature. Samples fixed in 77% ethanol were used for DNA and protein measurements. The cells were centrifuged, and for protein staining the cells were suspended in 50 mM potassium phosphate buffer (pH 7) containing 20 µM fluorescein isothiocyanate. After 60 min of incubation the cells were centrifuged and washed with the same buffer lacking fluorescein isothiocyanate. (It was found that this washing procedure eliminated an otherwise significant amount of nonspecific binding of the dye to the cells.) For DNA staining a combination of two dyes, ethidium bromide and mithramycin, was used to maximize both the specificity and the fluorescence yield (50); 20 µl of ethidium bromide and 200 µl of mithramycin (each from a 100-µg · ml<sup>−1</sup> stock solution) were added to a pellet of fixed cells, and the preparations were incubated for 15 to 20 min at room temperature and measured with the flow cytometer. For all measurements the cells were carefully dispersed before flow cytometry by repeated passage through a 0.4-mm needle to eliminate clumps.

To measure the fluorescence of the reduced (formazan) form of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) in the flow cytometer (19), a filter block with the following characteristics was used: excitation at 530 to 550 nm, band stop at 560 nm, and emission at ≥580 nm. The cells were incubated in 50 mM potassium phosphate (pH 7.4) supplemented with 4 mM CTC for 60 min at room temperature prior to the flow cytometric analysis.

Monodisperse (coefficient of variation, <2%) latex particles were obtained from Sigma or from Dyno Particles A/S, Lillestrøm, Norway.

**Respiratory activity.** The oxygen consumption rates of cells respiring endogenously were measured by using a Clark type oxygen electrode at 30°C in 50 mM potassium phosphate buffer (pH 7.4).

**Cell viability as assessed by plate counts.** Plates containing 1.3% nutrient broth E (LabM) were used to assess cell viability. Cell dilutions were made in quadruplicate by using lactate minimal medium (see above) lacking lactate. The cells were carefully dispersed by repeated passage through a 0.4-mm needle to eliminate clumping. The plates were incubated at 30°C for 3 days. (Although rich medium was used for these plates, we found that the levels of viability were no lower than the levels obtained when plates containing lactate minimal medium solidified with agar were used.)

**Total cell counts.** Unstained cells were counted with a phase-contrast microscope and an improved Neubauer counting chamber (18). At least 500 cells were counted in each case. This method has a level of precision better than ±6% (4a).

**Chemicals.** Rhodamine 123 and penicillin G were obtained from Sigma. Most other chemicals were of analytical grade and were obtained from Sigma or BDH; CTC was obtained from Polysciences, Eppeleheim, Germany.

## RESULTS

**Behavior of *M. luteus* cells in extended stationary phase.** After a batch culture of *M. luteus* cells had reached the stationary phase, further agitation during the first 10 days of starvation resulted in a decrease in the total cell concentration (by approximately 40%), a dramatic decrease in the culture's respiratory activity, and some decrease in the optical density of the culture (Fig. 1 and 2) Further storage of

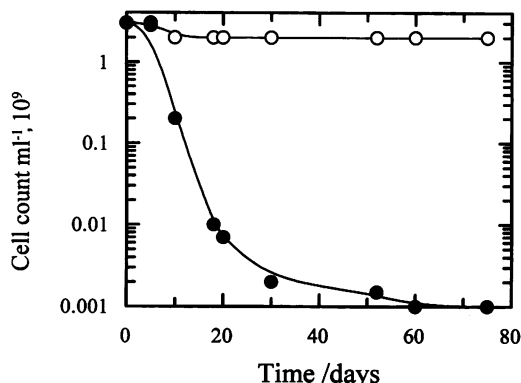


FIG. 1. Changes in the viable and total counts of *M. luteus* cells grown in batch culture and subjected to starvation. Cells were grown and starved and the total and viable counts were measured as described in Materials and Methods. Time zero corresponds to the onset of the stationary phase. Symbols: ○, total counts; ●, viable counts.

such cells resulted in minor but reproducible fluctuations in optical density, possibly reflecting cryptic growth, which stabilized after 1 month. There were no detectable changes in the total cell counts after 10 days of storage, while the viable counts decreased by more than 4 orders of magnitude from the initial viable counts.

Flow cytometric experiments (low-angle light scattering) revealed a rapid decrease in the mean cell diameter (from apparent values of 1.3 to 0.4  $\mu\text{m}$ ) after the cells entered the stationary phase, accompanied by a decrease in the relative standard deviation of this parameter, indicating that a more homogeneous population was present during storage (Fig.

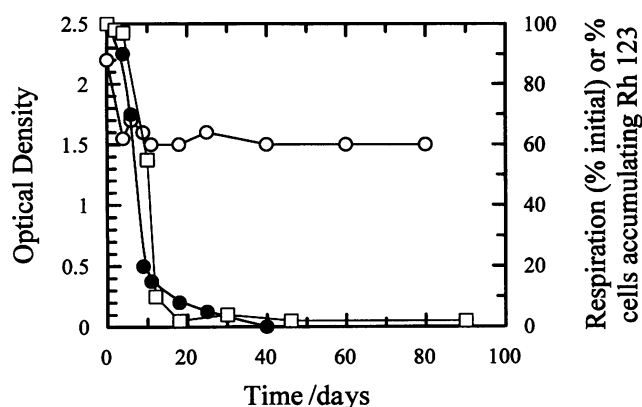


FIG. 2. Changes in the optical density, respiratory activity, and ability to accumulate rhodamine 123 (Rh 123) of *M. luteus* grown in batch culture and subjected to starvation. Cells were grown and starved and measurements were determined as described in Materials and Methods and the legend to Fig. 1. Respiratory activity was determined polarographically and was normalized; its initial value was equivalent to 150 natoms of  $\text{O} \cdot (\text{min} \cdot \text{mg} [\text{dry weight}]^{-1})^{-1}$ . Flow cytometry was performed by using a PMT voltage for the fluorescence channel of 650 V; this ensured that all of the initial fluorescence was sensitive to 15  $\mu\text{M}$  CCCP (18). Time zero corresponds to the onset of the stationary phase. Symbols: ○, optical density (path length, 0.35 mm); ●, respiratory rate; □, CCCP-sensitive rhodamine uptake into a flow cytometer channel above 14. The PMT voltage used for rhodamine 123 uptake was 650 V.

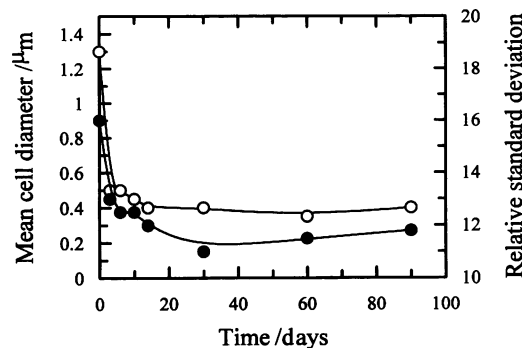


FIG. 3. Changes in the mean cell diameter and the relative standard deviation of the cell diameter for *M. luteus* grown in batch culture and subjected to starvation. The cells were grown and starved and the cell size distributions were measured as described in Materials and Methods. Time zero corresponds to the onset of the stationary phase. The PMT voltage for light-scattering measurements was 500 V. Symbols: ○, apparent cell diameter; ●, relative standard deviation.

3). The DNA content of the cells as judged by flow cytometry did not change even after 3 months of storage (Fig. 4). No evidence of the existence of a population of cells lacking DNA was found; indeed, the DNA distribution was less heterogeneous in a population of aged cells than in cells at the beginning of the stationary phase (Fig. 5). In contrast to the DNA content, the protein content of the cells decreased significantly, as shown by the decrease in the percentage of cells exhibiting detectable fluorescein isothiocyanate fluorescence (Fig. 4).

Along with the decrease in respiratory activity during starvation, we also observed a gradual decrease in the uncoupler-sensitive accumulation of rhodamine 123 (expressed as the percentage of cells exhibiting rhodamine fluorescence in channel numbers above 14 [Fig. 2] or in terms of the distribution of cell fluorescence [Fig. 6]). However, after even 20 days of starvation, only 2 to 5% of the small residual cell fluorescence (amplified in Fig. 6 by using a higher PMT voltage) was sensitive to the uncoupler

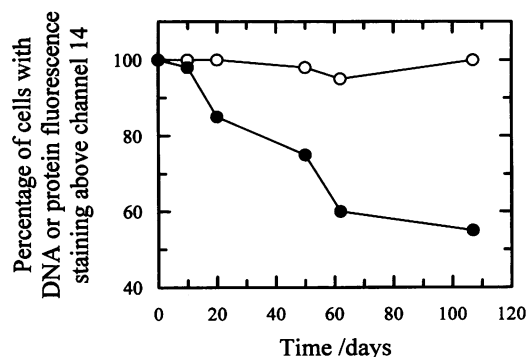


FIG. 4. Changes in the DNA and protein contents of *M. luteus* grown in batch culture and subjected to starvation. The cells were grown, starved, and stained for DNA and protein measurements as described in Materials and Methods. Relative changes were expressed as the percentage of cells which fluoresced in a channel number above 14. Time zero corresponds to the onset of the stationary phase. The PMT voltages for DNA (○) and protein (●) measurements were 600 and 750 V, respectively.

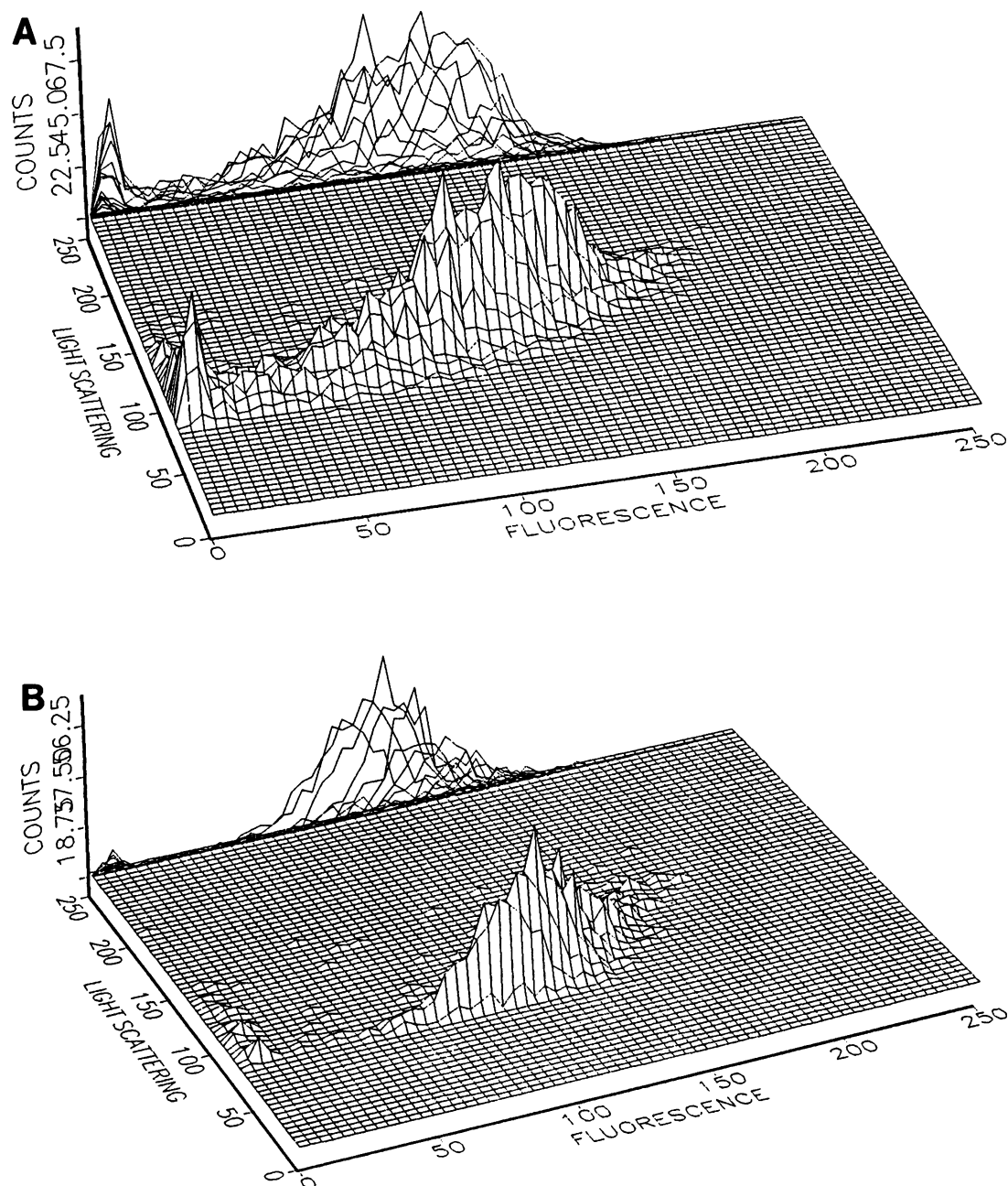


FIG. 5. Changes in DNA distribution in starving *M. luteus* cells. Cells were grown and starved as described in the legend to Fig. 1. Light scattering and the fluorescence of cells stained for DNA were measured flow cytometrically as described in Materials and Methods by using PMT voltages of 350 and 600 V for the light-scattering and fluorescence channels, respectively. The *x* and *y* axes represent the channel numbers of the flow cytometer; larger values indicate greater extents of light scattering or fluorescence. Signals from the flow cytometer were amplified logarithmically; full scale (going from channels 1 to 256) represented 3.5 decades of light scattering or fluorescence. The *z* axis represents the number of cells with the light-scattering and fluorescence properties indicated. Measurements were determined immediately following the onset of the stationary phase (A) and after 106 days of storage (B).

carbonyl cyanide *m*-chlorophenylhydrazine (CCCP). (If the 75-day-old cells were washed in minimal medium lacking L-lactate prior to measurement, some sensitivity [six channels] of the fluorescence distribution pattern to CCCP was detected, and this sensitivity was accompanied by an increase in viability of up to threefold.)

**Cell resuscitation.** Cells that had been starved as described

above for 75 days were washed in minimal medium lacking L-lactate and inoculated into fresh medium containing 0.5% L-lactate and 0.05% yeast extract. After 16 to 18 h of incubation at 30°C, almost parallel increases in total counts, viable counts, and the percentage of cells which accumulated rhodamine 123 in a CCCP-sensitive manner were observed (data not shown). Such findings clearly indicated

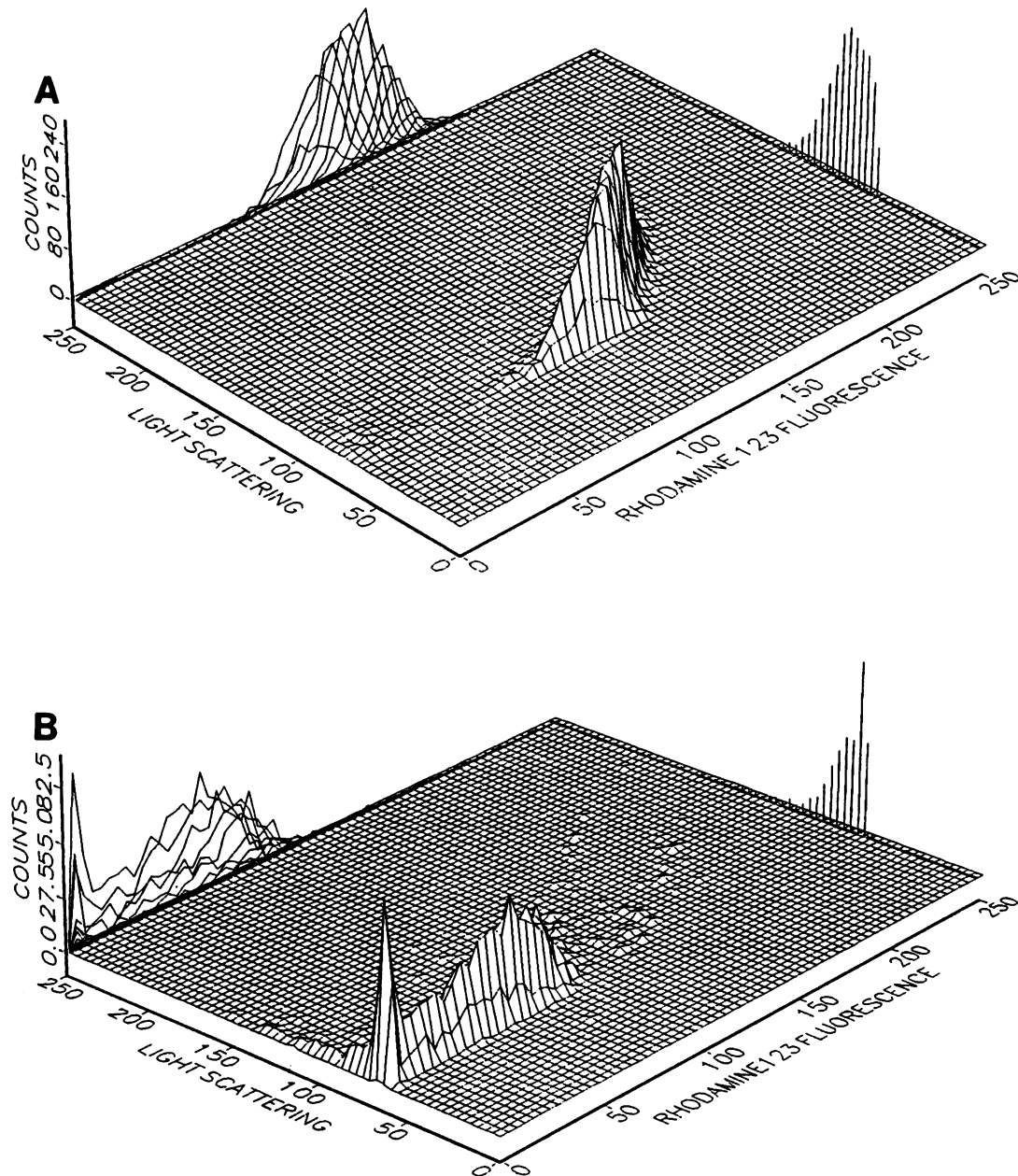


FIG. 6. Changes in the ability of *M. luteus* grown in batch culture and subjected to starvation to accumulate rhodamine 123. Cells were grown and starved and measurements were determined as described in Materials and Methods and the legend to Fig. 1. Flow cytometry was performed by using PMT voltages for the light-scattering and fluorescence channels of 500 and 750 V, respectively; the latter value meant that not all of the fluorescence was sensitive to 15  $\mu$ M CCCP (18). Measurements were determined immediately after the onset of the stationary phase (A) and after 90 days of storage (B).

the capability for growth of aged cells in culture; however, what kind of cells were responsible for the growth observed (i.e., whether a small percentage of colony-forming cells or the majority of the cells in the population were responsible for the growth observed) remained unclear. To overcome this uncertainty, we pretreated the cells with penicillin G, which should have resulted in the "killing" of any living cells in the population while dormant cells should not have been affected (4, 9), a particularly important point in studies of this type (54). Figure 7 shows that the apparent level of

viability of washed 2-month-old cells after 9 to 10 h of incubation in lactate medium containing yeast extract and penicillin G decreased by 2 orders of magnitude and did not change upon further incubation. After such a treatment of cells starved for 75 days and the removal of penicillin by washing, the cells were resuscitated in media containing different concentrations of lactate and yeast extract. The greatest level of resuscitation was obtained when lactate minimal medium supplemented with 0.5% L-lactate and 0.05% yeast extract was used for this. We found that after 20

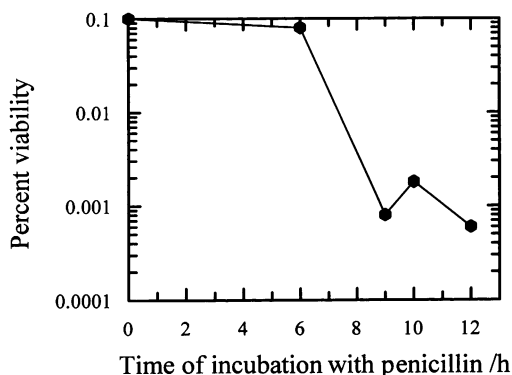


FIG. 7. Decrease in the viability of starved cells of *M. luteus* caused by incubation with penicillin G. Cells were grown and starved for 2 months and measurements were determined as described in Materials and Methods and in the legend to Fig. 1.

h of incubation in resuscitation medium, cells with the ability to exhibit CCCP-sensitive accumulation of rhodamine 123 appeared. This population of cells was very well differentiated from the rest of the cells by flow cytometry (Fig. 8). At the same time that there was an increase in the percentage of cells which accumulated rhodamine to an extent that caused them to fluoresce in channel numbers greater than 100, there was an increase in the percentage of cells which were able to accumulate significant amounts of the reduced form of CTC (a fluorescent formazan [51] which may be used to identify respiratory activity in situ [10, 44]) (Fig. 9). In this period the viable cell counts returned almost to the initial level (i.e., the level after 10 days of starvation [Fig. 1]), while the total cell counts remained constant within the limits of experimental error (Fig. 9 and 10). During the early phase of increasing viable cell counts, the percentage of cells that accumulated rhodamine and (after CTC staining) formazan was higher than the percentage of viable cells as determined by plating; later, these values were closer to each other.

As judged by flow cytometry (low-angle light scattering), cells with higher rhodamine concentrations also tended to be larger (Fig. 8). We also found (data not shown) that 30 h after the beginning of resuscitation the population had split into two subpopulations, consisting of cells with peak channels in the low-angle light-scattering distribution corresponding to apparent diameters of 0.4 and 2.2  $\mu\text{m}$ . CCCP-sensitive accumulation of rhodamine was associated mainly with the subpopulation of larger bacteria (Fig. 8). Toward the end of resuscitation (when the level of viability was at least 50%), the population became more homogeneous (as judged by a decrease in relative standard deviation) and the modal diameter of the cells then was about 1.0  $\mu\text{m}$  (i.e., close to the diameter of normal logarithmic-phase *M. luteus* cells) (data not shown). During the resuscitation phase, when the total cell counts were (by definition) constant, the percentage of small cells decreased to approximately 15 to 20% and there was an equivalent increase in the percentage of larger cells (Fig. 10). Finally, of course, following the resuscitation phase, the culture began to grow (Fig. 10).

When we performed a similar procedure to resuscitate *M. luteus* cells that had been stored for 7 months, we observed that approximately 25% of the population could be converted to a metabolically active state (larger cells which accumulated rhodamine 123) (Fig. 11); however, these cells

were neither able to produce colonies on agar plates nor able to grow in liquid media (data not shown).

## DISCUSSION

As has been observed with many bacteria (42, 43), lengthy storage of *M. luteus* in an extended stationary phase following batch culturing resulted in a decrease in the number of colony-forming cells. However, this decrease was not logarithmic, and the percentage of colony-forming units reached an apparently steady value after 60 to 80 days of storage of approximately 0.05% (Fig. 1). At the same time the total cell counts were maintained at a level that was 60 to 70% of the level at the beginning of the stationary phase, as has also been found for many bacteria starved in buffer or in water (23, 46). A similar form of "death curve" was found for *Aerobacter aerogenes* and was interpreted in terms of cryptic growth (42); however, in that case the "final" steady-state level of cultural viability (2 to 4%) was much higher than the level obtained in our study.

In our experiments, the population was rather unstable during the first 15 to 20 days of storage (as judged by variations in optical density and total counts [Fig. 2]); this apparently reflected the fluctuations that may be expected because of the simultaneous occurrence of synthetic and lytic processes in a culture (13). More recently, similar fluctuations were noted for psychrophilic marine bacteria starved in buffer (36). During this period of storage, a pronounced decrease in cell size also took place (accompanied by a decrease in protein content at a constant DNA content), as has been commonly observed for many bacteria when they are starved in buffer (10, 23, 47). Flow cytometry revealed that after 20 days of storage the cell population became much more homogeneous (in terms of size and DNA content) than the population at the beginning of the stationary phase (Fig. 5 and 6). We could not detect any cells with either significant respiratory activity (as judged either polarographically or by the capacity to reduce CTC to a fluorescent formazan) or an energized membrane (as judged by uncoupler-sensitive accumulation of rhodamine 123). However, we cannot exclude the possibility that cells stored for this amount of time (or more) could maintain a very small level of membrane energization.

Following the initiation of starvation in buffer, a very fast (hours) and significant decrease in the apparent proton motive force (as judged by the uptake of weak acids and lipophilic cations) was observed in *Thiobacillus acidophilus* (55), *Streptococcus cremoris* (39), and *Staphylococcus epidermidis* (15), while the marine bacterium *Vibrio fluvialis* maintained its apparent proton motive force at a constant level during 14 days of starvation (48). However, as discussed elsewhere (18, 22), it is not possible even in principle to correlate such macroscopic measurements with measurements of cell viability on plates.

It is clear that the great majority of cells in a population of *M. luteus* that has been stored for 20 days in the stationary phase (which are morphologically intact as judged by electron microscopy [data not shown]) persist in a state with undetectable metabolic activity. The changes in metabolism and in the chemical composition of the cells during this period remain unclear. At present we know only that the initial part of the stationary phase of *E. coli* is accompanied by the synthesis of new proteins and the expression of certain genes which are responsible for the survival of the cells (24, 47). While there has been much recent progress in understanding cognate processes in cells starved in nutrient-

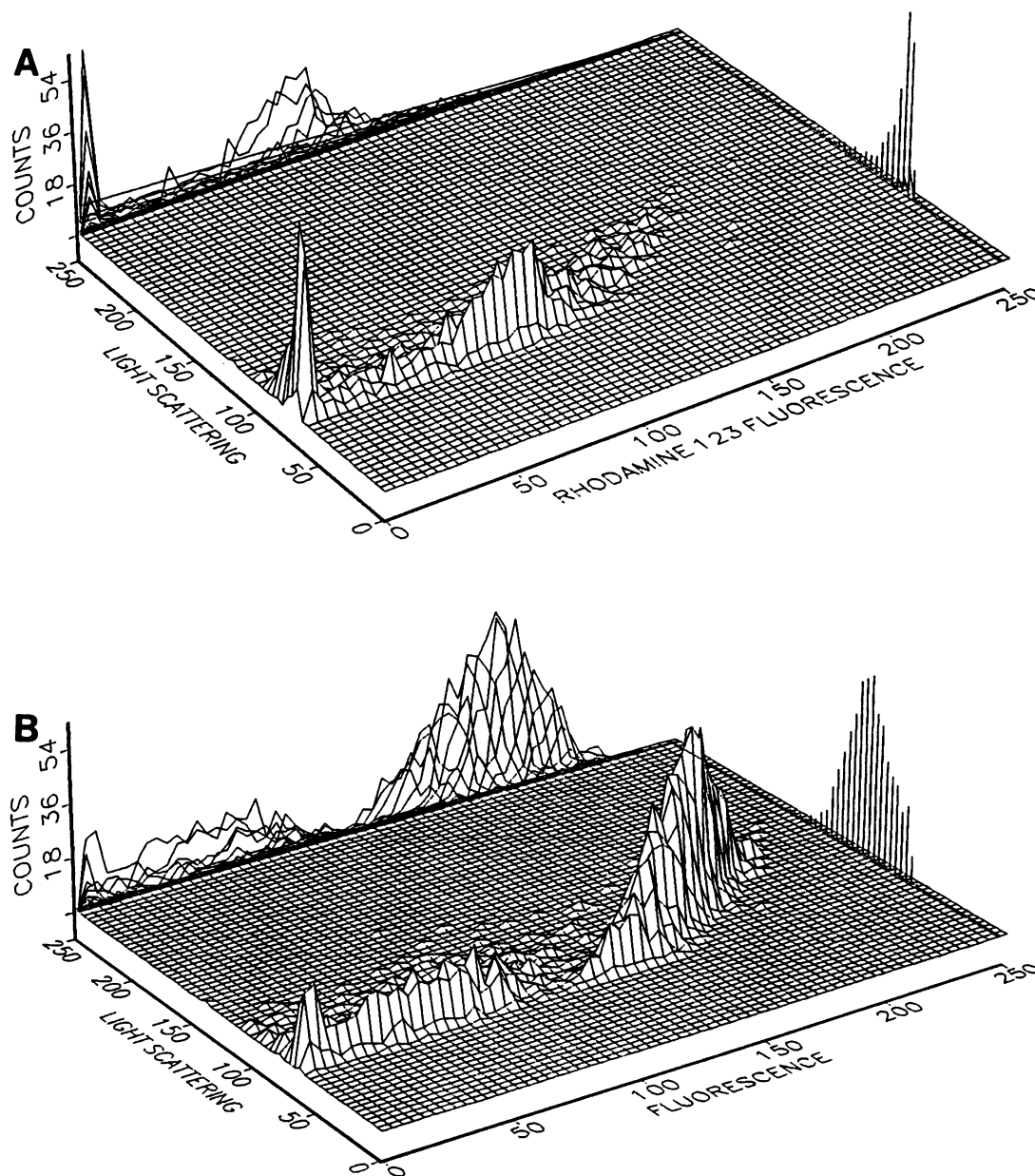


FIG. 8. Changes in light-scattering behavior and in the ability to accumulate rhodamine 123 during resuscitation of a starved culture of *M. luteus*. Cells were grown and starved and measurements were determined as described in Materials and Methods and in the legend to Fig. 1. Cells were starved for 75 days, incubated with penicillin G for 10 h, washed, and resuscitated as described in the text. Flow cytometry was performed by using PMT voltages for the light-scattering and fluorescence channels of 500 and 750 V, respectively. Data were obtained immediately after the penicillin treatment (A) and 45 h later (B).

free buffer or in water (23, 29–31), these studies did not deal with extended stationary phases such as those which we studied. In addition, it is worth stressing that all of the molecular genetic studies described in previous papers were carried out with cells which were able to grow on solid media, so that the properties of cells lacking this ability are not at all clear.

It is also worth mentioning that for stationary-phase cultures of *M. luteus* we found that approximately 10 h of storage is a critical point, after which the cells possess

significant stability. Storage of cultures in the stationary phase for only 2 h followed by washing in phosphate buffer led to massive lysis during the next 15 days, while cells which had first been starved for 20 h (or more) were stable under the same conditions for at least 2 months (data not shown). Perhaps such behavior reflects the synthesis of some stabilizing compounds during the early stages of storage of stationary-phase cultures (8, 24).

The existence in starved populations of actively growing bacteria is an extremely serious problem for studies of



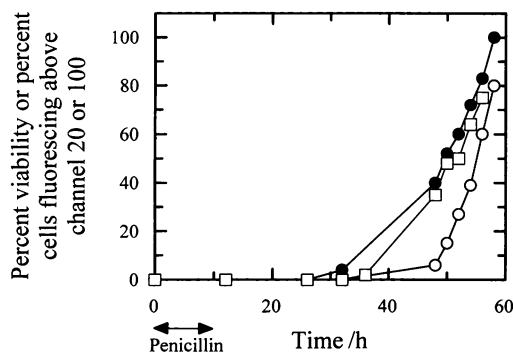


FIG. 9. Changes in viability, in respiratory activity, and in the ability to accumulate rhodamine 123 during resuscitation of a starved culture of *M. luteus*. Cells were grown and starved and measurements were determined as described in Materials and Methods and in the legend to Fig. 1. Cells were starved for 75 days, incubated with penicillin G for 10 h, washed, and resuscitated as described in the text. Flow cytometry was performed by using PMT voltages for the rhodamine and CTC (formazan) fluorescence channels of 750 and 650 V, respectively. Symbols:  $\circ$ , level of viability;  $\bullet$ , percentage of cells fluorescing in a channel number above 100 when the preparation was stained with rhodamine 123;  $\square$ , percentage of cells fluorescing in a channel number above 20 when the preparation was stained with CTC.

resuscitation (33, 37, 45, 54) and other processes (2, 9, 26, 32). We used pretreatment of the culture with penicillin to overcome this difficulty. Stabilization of the viable counts in a starved culture after 8 to 9 h of incubation with penicillin (Fig. 7) indicated that dividing cells were absent after the penicillin treatment. Only a very limited number of dormant cells (equivalent to a level of viability of less than  $10^{-5}$  at the end of the penicillin treatment) were able to multiply on solid agar medium. When this culture was washed to remove the penicillin and incubated with lactate and a low concentration of yeast extract, a population of larger cells appeared, which was able to accumulate rhodamine and to reduce CTC (similar differentiation of *M. luteus* cultures as judged by the first two criteria was also observed when cells were grown in a lactate-limited chemostat at a low dilution rate [18]).

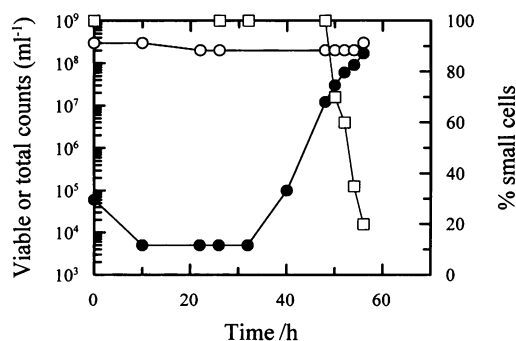


FIG. 10. Changes in viable and total counts and the percentage of small cells during resuscitation of a starved culture of *M. luteus*. Cells were grown and starved and measurements were determined as described in Materials and Methods and in the legend to Fig. 9. Cells were starved for 75 days, incubated with penicillin G for 10 h, washed, and resuscitated as described in the text. Symbols:  $\circ$ , total counts;  $\bullet$ , viable counts;  $\square$ , percentage of small cells (diameter,  $<0.5 \mu\text{m}$ ).

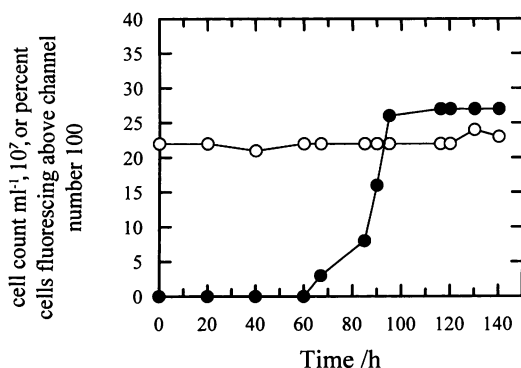


FIG. 11. Changes in total cell number and in the ability to accumulate rhodamine 123 during resuscitation of a starved culture of *M. luteus*. Cells were grown and starved and measurements were determined as described in Materials and Methods and in the legend to Fig. 1. Cells were starved for 7 months, washed, and resuscitated as described in the text. Flow cytometry was performed by using a PMT voltage for the rhodamine fluorescence channel of 750 V. Symbols:  $\bullet$ , percentage of cells fluorescing in a channel number above 100 when the preparation was stained with rhodamine 123;  $\circ$ , total cell counts.

Similarly, there was an increase in the viable counts when the total counts were constant. Quantitatively, during the first 20 h of resuscitation (while the cells were becoming larger), there was a significant difference in viability, as judged by plating and the percentage of metabolically active cells present as determined by flow cytometry (Fig. 9); the metabolically active fraction was apparently larger. This suggests that membrane energization is a necessary but not sufficient condition for resuscitating *M. luteus* cells so that they have the ability to multiply on solid media. It is possible that a small part of this difference (particularly at the end of resuscitation) can be accounted for by an underestimation of the level of viable cells as judged by plating, because of the tendency of cells to aggregate.

In principle, the increase of in the number of colony-forming cells in the population during resuscitation could be due to the growth of some cells at the expense of other cells in the population (cannibalism or cryptic growth). However, we stress that this possibility seems unlikely for the following reasons: (i) given the constancy of the total counts, it is implausible that lysis could be so exactly balanced by growth; (ii) during resuscitation the percentage of small cells in the population decreased and there was an exactly equivalent increase in the percentage of larger cells (Fig. 10), indicating that small, dormant cells were converted into larger, active cells; (iii) the transient character of the accumulation of larger cells during resuscitation (data not shown) suggests that the increase in the level of viable cells in this period was not due to typical growth but was due to an unbalanced increase in cell size during this period; and (iv) even if all of the cells at 30 h ( $5 \times 10^3$  cells per ml, [Fig. 10]) started to grow with a doubling time of 4 h (the approximate doubling time for normal cultures of *M. luteus* in this medium), the increase in viable counts after an additional 20 h (at the end of the resuscitation) should have resulted in only approximately  $1.6 \times 10^5$  cells per ml (while in the experiment shown in Fig. 10 the actual level was more than  $10^8$  cells per ml). We may put this important, quantitative argument (cf. the studies of Nilsson et al. [37] and Weichert et al. [54], in which only plate counts, not total cell counts,



were determined) in another way: in our case we went from a level of viability of essentially zero (as construed on a linear scale) to a level of viability of more than 50% at constant cell numbers (constancy was judged by a method whose precision was much better than  $\pm 6\%$ ). This argument alone excludes all relevance of any cells which might have remained in a viable (but penicillin-resistant) state after the penicillin treatment and which therefore might have exhibited merely apparent resuscitation (54).

We conclude that storage of *M. luteus* cells in the stationary phase for up to 2.5 months resulted in the transition of the cells to a dormant state from which at least 50% of the cells could be resuscitated. If the cells were stored in this way for 7 months, only 25% of the cells could be converted to a metabolically active state (as judged with the flow cytometer by light scattering, rhodamine uptake, and CTC reduction), but these cells did not form colonies on agar. These cells seem to resemble more closely cultures starved in buffers and in water in experiments in which the "direct viable count" method is used (3). It is of course not known whether such cells are genuinely unable to divide under any conditions or whether we have simply been unable to identify the proper conditions for resuscitation. Also not known is what it is about incubation in a liquid medium that allows cells to be resuscitated and ultimately to grow and thus differentiates it from incubation in the same medium solidified with agar.

Finally, we do not know how widespread the behavior that we have observed in stationary-phase cultures of *M. luteus* is among other nonsporulating, copiotrophic bacteria. However, the results obtained in this work open the possibility of studying dormant, vegetative cells in preparative amounts and at the same time raise important questions about the correct interpretation of the death phase of bacterial cultures as usually described in textbooks. If the ability to form a colony is the sole criterion of whether a cell is alive, it is reasonable to suggest that dormancy is likely to be far more common than death in stationary-phase microbial cultures.

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