DORMANCY IN NON-SPORULATING BACTERIA: ITS SIGNIFICANCE FOR ENVIRONMENTAL MONITORING

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Abstract

In natural ecosystems, the total cell count obtained microscopically typically exceeds the viable count on non-selective media by orders of magnitude. The question therefore arises as to whether the "invisible", apparently nonculturable cells are dead, are killed by our isolation media, or are merely in a dormant state from which we might in principle be able to resuscitate them if only we knew how. In particular the suggested "viable-but-nonculturable" (VBNC) bacteria have been invoked to explain phenomena as divergent as the epidemiology of some infections and the persistence of genetically marked organisms in the environment (e.g. the failure to isolate Vibrio cholerae and Campylobacter jejuni from clearly implicated sources or reservoirs of infection could be accounted for on the basis of their being present in a VBNC or dormant state). Application of flow cytometry may be a useful tool to visualize bacteria without their growing and to discriminate between dead and dormant bacteria. We found that dormant Micrococcus luteus can be physically separated from dead cells by flow cytometry sorter after cell staining with rhodamine 123. Resuscitation of dormant M.luteus cells in liquid medium does not proceed in the absence of a culture supernatant from batch-grown cells. This suggests that viable cells can excrete a pheromone-like substance necessary for the resuscitation of dormant cells.

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1. Introduction

We have established that in the case of complete exhaustion of exogenous nutrients bacteria can go into an anabiotic (dormant) state, which helps the cells to survive for a long time without growth and multiplication. Dormancy may be defined as "*a reversible state of low metabolic activity, in which cells can persist for extended periods without division*" [1]; it 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 one in which they are not "dead" in that when conditions are more favorable they can revert (by a procedure referred to as resuscitation) to a state of "aliveness" as so defined. The adoption by vegetative, nonsporulating bacteria of such dormant forms in natural environments is a subject of intense current interest (see e.g. [1-9]), and it has been suggested that such dormant bacteria may be represented by ultramicrobacteria [5] or the so-called viable-but-non-culturable (VBNC) forms [2] found in marine or river habitats (but cf. [1]). Although a characteristic property of dormant forms is their inability to produce colonies when plated *directly* onto solid agar media [1] the "VBNC" cells are usually taken to be revealed *via* by their metabolic activity [4] using the direct viable count technique [9].

It is increasingly evident that dormant (or nonculturable) forms are an important feature of hitherto-unrecognized disease states [10,11], and a gigantic biodiversity of both scientific and biotechnological interest [12], and their existence raises important questions concerning the famous Koch's postulates of microbial pathogenesis [13].

Foremost amongst the epidemiological mysteries are cholera and campylobacteriosis where the failure to isolate *Vibrio cholerae* and *Campylobacter jejuni* from clearly implicated sources or reservoirs of infection could be accounted for on the basis of their being present in a VBNC state. For both these organisms, environmental investigations have provided evidence for the presence of "nonculturable" cells in appropriate samples [14,15] while *in vitro* studies have demonstrated their capacity to form metabolically active cells which could not be grown immediately [16,17].

Thus there are many practical issues bearing on food and water safety, the distribution and influence of bacteria in the environment, the effects of antibiotics and the significance of declining colony-forming unit (cfu) counts which cannot be assessed until the authenticity of the putative dormant or VBNC state has been confirmed and how widely dormant cells are distributed. If some bacteria can differentiate into a "nonculturable" state, this undermines interpretation of all applications based on colony counting [4]. As discussed elsewhere [18,19], it is inevitable that the use of nucleic acid probe technology will reveal that many more diseases than are currently recognized do have a microbial aetiology [10,11], even when culture-based methods have not yet confirmed this [13].

The list of bacterial species which are claimed to be in dormant state, and where the apparent resuscitation of "nonculturable" cells has been reported is substantial (including such important Gram-negative pathogens as *Vibrio cholerae, Legionella pneumophila, Yersinia ruckeri, Salmonella enteritidis, Campylobacter jejuni, Helicobacter pylori* - Table 1), but the crucial question of whether such "dormant" forms could be converted to normal, viable cells remains open, despite the many experimental attempts that have been made to resolve it [1,20]. The central point of discussion in this area is now focused on the results of recovery or resuscitation experiments, almost all of which were done by cultivation of nonculturable cells on liquid media followed by plating onto agar plates. In the majority of studies, recovery has been difficult both to produce and to reproduce. Although some early experiments have purported to show the ability of "nonculturable" bacteria to grow on agar following resuscitation *in vitro* in appropriate liquid media, a limited number of operationally viable cells in the starved population could have been responsible for the growth which occurred. Indeed, in almost all published cases the populations of nonculturable bacteria diluted to an extent which might have been sufficient, statistically, to remove any viable cells, were not able to be resuscitated (Table 1) [21].

TABLE 1. A summary of some studies in which resuscitation of 'dormant' or a 'nonculturable' bacterium has been attempted.

Aeromonas salmonicida	starvation in sea water, 15°C	+	-	usage of rich medium (TSB) for resuscitation	[22]
Aeromonas salmonicida	starvation in sea water, 4°C	-	-	various media and conditions have been used for resuscitation	[20]
Aeromonas salmonicida	starvation in water, 10°C	-	+	various resuscitation media have been used	[23,24]
Campylobacter jejuni	starvation in physiological saline solution, 20°C	-	-	resuscitation in simulated stomach, ileal and colon environments (rich media)	[25]
Campylobacter jejuni	starvation in sterilized pond water, 4°C	+	-	resuscitation of some strains via passage in mice	[26]
Klebsiella pneumoniae	starved bacteria in phosphate buffer	+	-		[27]
Legionella pneumophila	starvation in pure water, 30°C	-	-	resuscitation in co-cultures with *T. pyriformis*	[28]
Micrococcus luteus	long storage in stationary phase, room temperature	+	+	resuscitation factor (supernate taken from active culture) required	[29]
Pasteurella piscicida	starvation in seawater, 6 and 20°C	+	-		[30]
Salmonella enteritidis	starvation in salt solutions, 21°C	-	+	usage of lactose broth (Difco) for resuscitation	[31]
Salmonella enteritidis	starvation in sterilized river water, 25°C	+	-	resuscitation by nutrient addition after 4 but not 21 days after culturability lost	[32]

TABLE 1. Cont'd

Pseudomonas fluorescens	starvation in soil, 24°C	+?	+	only several divisions of "VBNC" cells during resuscitation were found	[33]
Pseudomonas fluorescens	N-starvation in minimal medium, 25°C	+	-	usage of medium lacking a carbon source for resuscitation	[34]
Vibrio cholerae	starvation in autoclaved water	+	-	usage of passage through rabbit ileal loop	[35]
Escherichia coli *Vibrio cholerae*	starvation in autoclaved artificial sea water, 4°C	-	+	usage of nutrient- free medium for resuscitation	[36]
Vibrio, Aeromonas, Pseudomonas, Alcaligenes spp.	non-culturable ultramicrobac-teria from estuarine waters	+	-	resuscitation was found for a narrow range of nutrient concentrations	[37]
Vibrio parahaemolyticus	starvation in mineral medium under 3.5°C	-	+	usage of rich medium for resuscitation	[38]
Vibrio cholerae	starvation in salt solution , 15°C	+	‡‡	Conversion to the colony- forming cells was effected with a short heat shock	[39]
Vibrio cholerae	starvation in buffered saline, 4°C	+/-	+/-	Resuscitation in intestine after ingestion of non-pathogenic vaccine strains by volunteers. 2 sets of experiments were done; only one claimed resuscitation. Dilutions were probably not great enough to exclude presence of some viable cells	[40]
Vibrio vulnificus	starvation in defined media, 5°C	+	-	usage of natural estuarine environment for resuscitation	[41]

TABLE 1. Cont'd

Vibrio vulnificus	starvation in defined media, 5°C	+	+|	*in vivo* resuscitation (injection in mice)	[42]
Vibrio vulnificus	starvation in defined media, 5°C	-	+	wide range of conditions for resuscitation *in vitro* were used	[43]
Yersinia ruckeri	starvation in sterile river water, 6 or 18°C	+	-	usage of rich medium for resuscitation	44]

" +" sign (third column) means that resuscitation was attempted and indeed claimed as judged by the appearance of increased numbers of culturable organisms, whilst the - sign means that no recovery or resuscitation was observed.

"+" sign (fourth column) means that the author(s) diluted the samples before performing resuscitation, in an attempt to remove genuinely viable cells present at the start of the resuscitation experiment, whilst a - sign means that they did not.

| The growth of cells on the medium used here may have underestimated the culturable fraction, since there is evidence that this system contains an injured fraction [43].

‡‡ Dilutions *per se* were not done but at one stage *no* viability was observed, although the resuscitation yielded 1000 colonies (again the total count of bacteria at the onset of resuscitation was not shown).

The difficulties in testing for the presence of dormant forms in environmental samples by conventional methods (due to their inability to grow on solid or liquid media) make the development of new approaches to detect bacteria in dormant states an important task for epidemiology and environmental monitoring.

2. Results

In recent work, we have found that cells of the nonsporulating, copiotrophic, Gram-positive coccus *Micrococcus luteus* starved for 3-7 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 viability ($< 10^{-4}$) as estimated by plating on agar plates, while the total count remained close to its initial value. Using flow cytometry with appropriate probes and conditions we found that a high percentage of *M. luteus* cells in 3-month-old populations could be resuscitated to normal, colony-forming bacteria [8], 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 [29]. These and other data led 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.

However the assessment of the percentage of resuscitable cells in a population could, on the basis of these and related experiments, only be made *after* cell resuscitation where, under some conditions, an excellent correlation could be found between the *percentage* of dormant cells and the different extent to which rhodamine 123 could be accumulated in an uncoupler-sensitive fashion by different cohorts of cells. 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 or not. 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 physically to separate subpopulations of cells with different properties for their further examination.

Figure 1A show the typical distribution of the fluorescence of non-starved *M. luteus* cells that had been stained with the membrane energization-sensitive cation Rh123 and studied in the flow cytometer. We discriminated the extent of staining as follows. Channel 136 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 (Figure 1A), which is fully uncoupler sensitive (not shown). Figure 1B shows a typical distribution of the fluorescence of *M. luteus* cells that had been starved for 5 months, stained with Rh123 and studied in the flow cytometer. 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 followed by treatment with a suitable concentration of the uncoupler CCCP

exhibited a fluorescence in this region. Although starved cells in region B (between channels 80 and 136) of Figure 1B had an elevated fluorescence relative to those in region A, its sensitivity to CCCP was also very low (only 2-5% of the cells in region B exhibiting a decrease in fluorescence after CCCP treatment).

FIGURE 1. Distribution of the fluorescence of non-starved (a) and starved (b) 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 and resuspended in lactate minimal medium lacking lactate and stained with Rh123 (0.3 mM) (a), 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 using the Skatron Argus 100 instrument for the ability to accumulate Rh123.

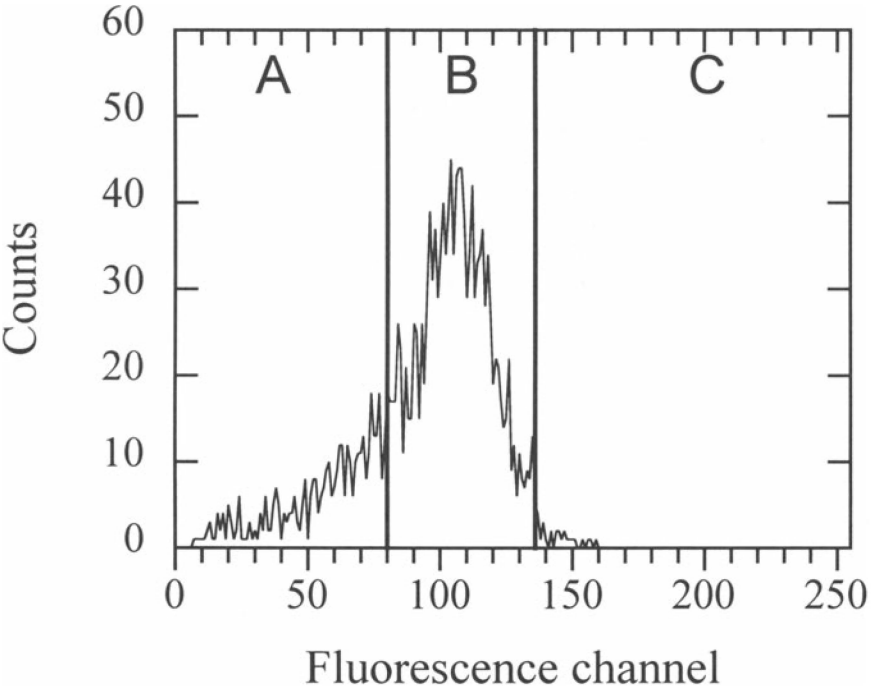


Figure 1A

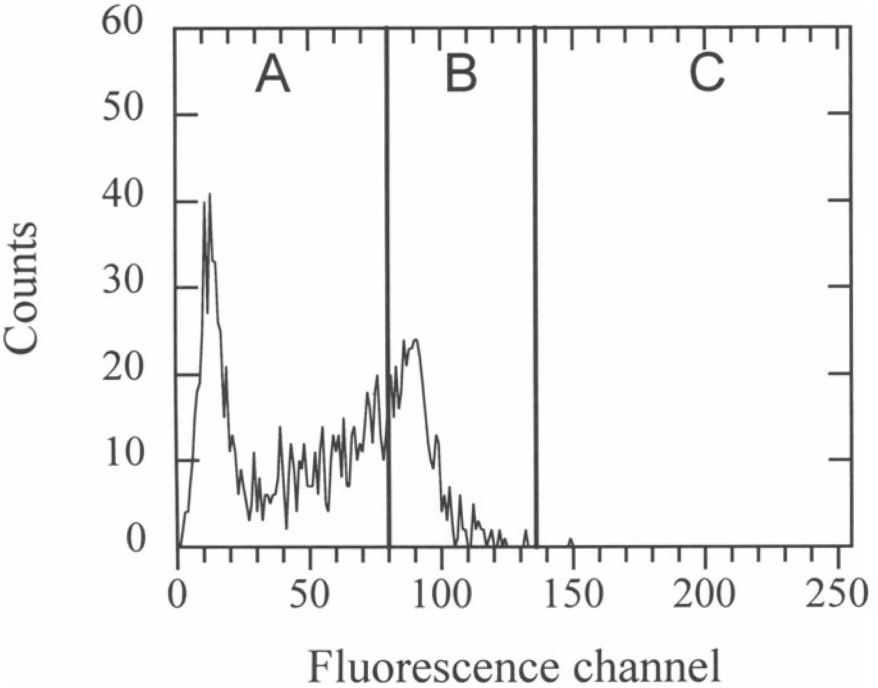


Figure 1B

We sorted cultures whose fluorescence was of the type displayed in Figure 1B and Figure 2 into two populations: (i) cells of which the rhodamine staining was sensitive or partially sensitive to CCCP (regions B+C of Figure 1) and cells whose rhodamine-dependent fluorescence was not sensitive to CCCP (region A of Figure 1). 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 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+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+C and in the whole population after sorting (per 10^6 cells) was unexpectedly and significantly more (8-20 times in different experiments) than those before sorting. Similar results were obtained using plates with lactate minimal medium (not shown).

FIGURE 2. Distribution of *M. luteus* cells that had been starved for 5 months and stained with rhodamine 123 and subjected to sorting in a flow cytometric cell sorter. For experimental details see legend to Figure 1. The horizontal line shows the sorting region which was used to discriminate between regions A and B+C. Cells from the 2 regions were sorted differentially into separate test tubes, and examined by MPN assay, plating and assessed for their total counts. The scales are logarithmic and cover 4 decades.

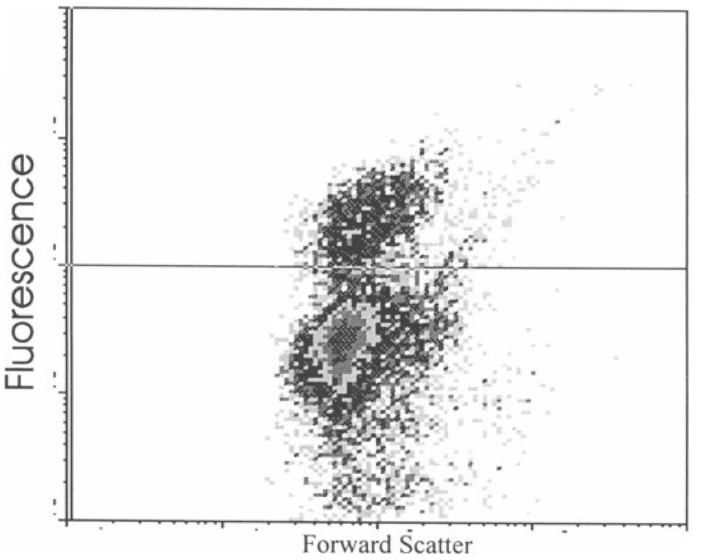


TABLE 2. Total count, viable count and the distribution in viability of cells taken from cultures of *M. luteus* that had been starved for 5 months, before and after cell flow cytometric analysis.

	sorting			
	before sorting	after sorting		
		region A	region B+C	regionA+B+C
total count	10^6	$8.\ 10^5$	2.10^5	10^6
viable count by plates	740	42	6200	6240
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 the presence of supernatant)	3%	0.004%	19.3%	3.9%

The distribution of the total count between regions A,B and C was estimated from the flow cytometric distribution pattern (as in Figure 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 the count of viable bacteria on agar plates (second row). The viable count and hence viability by MPN assay was performed as described in Materials and Methods save that lactate minimal medium with 0.05% yeast extract for assay was used without (4th row) or with (5th 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.

We used the most probable number (MPN) method, which allows one to estimate viable cell numbers by their cultivation in liquid medium at high dilution [29]. The basic idea of such a measurement is that provided that the MPN medium supports resuscitation as well as growth, a dormant cell would score as viable, in contrast to its behavior as judged by direct plating. Under these conditions the growth of a limited number of viable cells during resuscitation is excluded. Appropriate resuscitation medium contained lactate minimal medium + yeast extract + supernatant from batch cultures of *M. luteus* grown in lactate minimal medium. The MPN assay was performed in a Bioscreen C optical growth analyzer (Labsystems, Finland). For the MPN assay supernatant taken from a batch culture of the organism grown in LMM (plus 0.5% lactate) to an OD of 2, slightly before the beginning of stationary phase, was added at a concentration of 0.1 ml of supernatant per well containing 0.1ml resuscitation medium with different concentration of yeast extract and starved cells. Under these conditions, we *did* observe cell growth in inocula taken from starved cells suspensions at very high dilutions (10^7- to 10^{10}-fold) after incubation of the test tubes for 72-120 h (Table3)

TABLE 3. Resuscitation of dormant *m. luteus* cells in liquid medium.

N culture	time of starvation	total count	viability by cfu	viability by MPN
1	2 months	$5.3\ 10^9$	$5\ 10^6$	$3.5\ 10^9$
2	4.5 months	10^{10}	$1.3\ 10^6$	$9.2\ 10^9$
3	6 months	$1.2\ 10^{10}$	$3.6\ 10^4$	$9.2\ 10^9$
4	9 months	$6.2\ 10^9$	$5.2\ 10^5$	$5.4\ 10^9$

The important condition for such an estimation was to add diluted supernatant from growing bacteria to the media in which the MPN assay was performed. We found that the effect of supernatant on the resuscitation of dormant cells was maximal when the supernatant had been taken from the late logarithmic phase of batch-grown bacteria (not shown). The active fraction from supernatant was purified by a combination of anion exchange media, and the final activity was eluted from a linear KCl gradient on a MonoQ column. This substance is a protein with a molecular mass of ca 17 kDa, which in concentrations of several ng/ml results in the resuscitation of starved cells and increases the viability of these culture at least 100-fold (Figure 3). This protein also stimulates the growth of viable *M. luteus* cells, and can therefore be considered as a growth factor or bacterial cytokine.

Table 2 demonstrates the resuscitation of cells under such conditions for the whole population before sorting (showing an increase in the viable count when MPN was done in the presence of supernatants by some 50-fold in these cultures that had been starved for 5 months). The same effect was observed when cells from sorted from regions B+C were subjected to resuscitation, while resuscitation of cells from region A was unsuccessful (Table 1).

For additional discrimination between different type of cells in starved population we monitored the state of the permeability barrier of the cells by staining with propidium iodide (PI, a DNA staining probe). We established that this probe does not penetrate through the cytoplasmic membrane of intact *M. luteus*, while octanol administration to the cell suspension (0.5%) resulted in 100% stained cells (Fig 4). Observation of different starved cultures of *M. luteus* revealed that in some cultures where the percentage of PI-positive cells is close to 100% the resuscitation of cells was not successful (even in the presence of supernatant). This can indicate the correlation between state of permeability barrier and ability of starved cells for recovery.

3. Discussion

In the present study starved cultures revealed two different type of cells, with low and elevated fluorescence. The fluorescence intensity of cells in population B was comparable with the fluorescence of (actively growing) *M. luteus* cells, although in the case of 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 12) which could results in increased Rh123 adsorption or fluorescence quantum yield by the cells. Indeed, a characteristic property of starved bacteria (see 4) is an increase in their surface hydrophobicity.

FIGURE 3. Effect of purified 17-kDa protein on the resuscitation of dormant cells of *Micrococcus luteus*. *M. luteus* was starved and its viability assessed using an MPN assay as described [Kaprelyants et al 1994 FEMS]. Supernatants were mixed in the stated proportion with a lactate minimal medium containing 0.05 (w/v) yeast extract and used in the MPN assay.

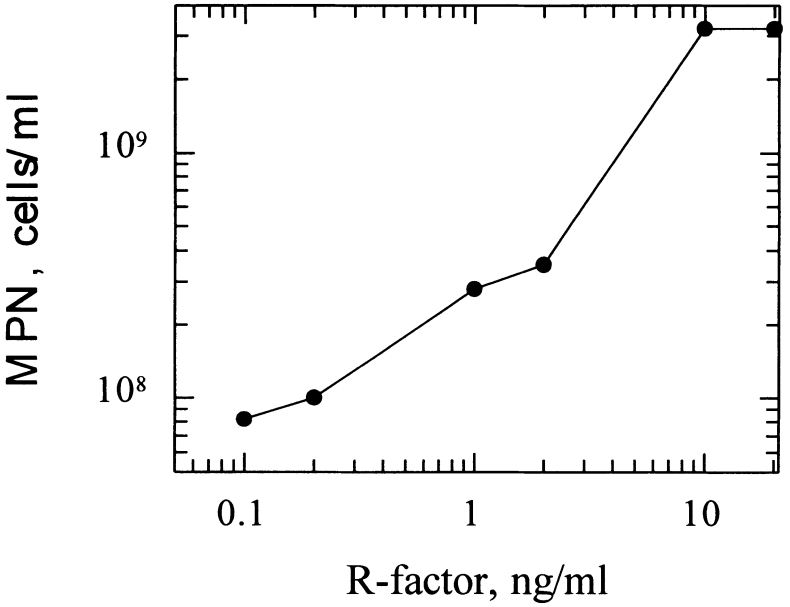
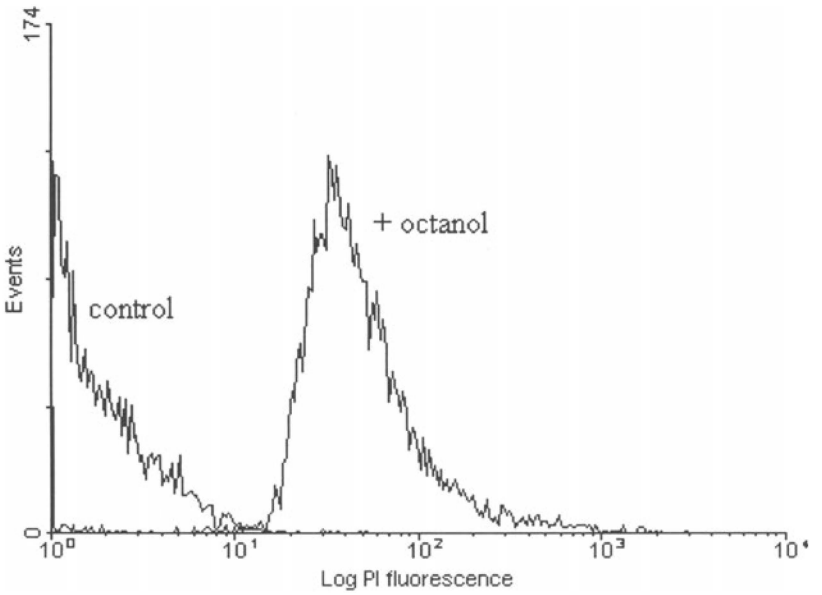


FIGURE 4. Distribution of the fluorescence of starved for 5 months cells of *M. luteus* stained with propidium iodide and assessed by flow cytometry. For experimental details see legend to Figure 1. Octanol was added to the cells before measuring to final concentration of 0.2%.



Cell sorting revealed that the viable cells in cultures starved for 5 months are concentrated mainly in regions B and C (Figure 1B). The viable count of the whole culture, as well as the population in regions B+C, increased after cell sorting. This result can be rationalized in the light of the existence of a "killer factor" which can be produced by starved cells during their resuscitation (13). The resuscitation of cells as judged by the MPN assay was successful for cells in regions B+C but not for cells in region A. This constitutes direct evidence that dormant cells are concentrated in regions B+C.

In conclusion, populations of the nonsporulating bacterium *M. luteus* that have been starved for an extended period are 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 active protein found in this study is to our knowledge the first purification of a factor which stimulates the resuscitation of bacteria after true dormancy. Since the factor could also stimulate the growth rate of viable cells, it is very likely that this protein is involved in the normal control of cell multiplication, although the molecular mechanism by which this factor facilitates resuscitation remains to be understood. We would also comment

on the implications of the self-promoting mode of cell growth and recovery after dormancy for medicine and environmental microbiology, especially in the case of infections in which bacterial cells may persist for extended periods in latent or dormant states.

The two subpopulations of starved culture of *M .luteus* could be discriminated and sorted out from the latter on the basis of their ability to bind the weakly lipophilic cation dye Rh123 and to be stained by the membrane-impermeable propidium iodide. Flow cytometry is a powerful method for the analysis of heterogeneity in bacterial systems and promising tool for environmental monitoring.

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