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Estimation of dormant *Micrococcus luteus* cells by penicillin lysis and by resuscitation in cell-free spent culture medium at high dilution

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Abstract: Micrococcus luteus starved for 2–7 months in spent medium following growth to stationary phase in batch culture exhibited a culturability (as estimated by direct plating on nutrient agar plates) of $< 0.001\%$. However, following a lag, some 70% of the cells could be lysed upon inoculation into and cultivation in fresh lactate minimal medium containing penicillin, showing the capability of a significant portion of the cells at least to enlarge (and thus potentially to resuscitate). When the viable cell count was estimated using the most probable number method, by incubation of high dilutions of starved cells in liquid growth media, the number of culturable or resuscitable cells was very low, and little different from the viable cell count as assessed by plating on solid media. However, the apparent viability of these populations evidenced with the most probable number method was 1000–100000-fold greater when samples were diluted into liquid media containing supernatants taken from the stationary phase of batch cultures of the organism, suggesting that viable cells can produce a factor which stimulates the resuscitation of dormant cells. Both approaches show, under conditions in which the growth of a limited number of viable cells during resuscitation is excluded, that a significant portion of the apparently non-viable cell population in an extended stationary phase is dormant, and not dead.

Key words: Micrococcus luteus; Stationary phase; Dormancy; Resuscitation; Cryptobiosis; Viable counting

Introduction

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 favourable they can revert (by a process referred to as resuscitation) to a state wherein they can grow and replicate as normal. Resuscitation often involves preincubation in a suitably weak liquid nutrient medium. The adoption by vegetative, non-sporulating bacteria of such dormant forms in natural environments is a subject of intense current interest (see e.g. [1–5]), and it has been suggested that such dormant bacteria may be represented by ultramicrobacteria [6] or the so-called viable-but-non-culturable (VBNC) forms [2] found in marine or freshwater

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habitats (but cf. [1]). Although a characteristic property of these forms is their inability to produce colonies when plated directly on solid agar media [1,7–9], VBNC cells can manifest themselves by their metabolic activity (i.e. substrate responsiveness) as disclosed by the so-called direct viable count technique [10].

In fact, there is presently little strong evidence that ultramicrobacteria or VNBC are truly dormant. By definition, the dormant state must be reversible, such that the only 'benchmark' criterion for dormancy is the ability of bacterial cells to multiply on an appropriate medium following suitable resuscitation. Although some experiments have shown the ability of bacteria presumed to be dormant to grow on agar following resuscitation in liquid media [11–14], it has been emphasised [15,16] that a limited number of genuinely viable cells in the starved population could have been responsible for such growth as occurred. Indeed, it has been shown that populations of non-culturable *Aeromonas salmonicida* [15,16] or *Vibrio vulnificus* [17] diluted to an extent which might have been sufficient to remove any viable cells were not amenable to resuscitation. Recently, however, using flow cytometry to monitor the behaviour of individual cells, we have found that a significant fraction of the population of *Micrococcus luteus* cultures definitely adopted a dormant state when grown in carbon-limited chemostat at low dilution rate [7,8], or when incubated in spent growth medium in a lengthy stationary phase following batch growth, when the apparent viability had decreased to some 0.001% as judged by plate counts on solid media [9]. In particular, by monitoring populations of *M. luteus* starved in the stationary phase of growth, and by killing viable cells using penicillin prior to resuscitation, we obtained strong evidence that 50% or more of the cells in the population were dormant [1,9]. Here, using similar populations, we describe two approaches for the estimation of dormant bacteria under conditions which exclude possible bacterial regrowth during resuscitation. We find, as before, that high concentrations of dormant bacterial cells can be sustained in the stationary phase following batch growth.

Materials and Methods

Organism and media

Micrococcus luteus Fleming strain 2665 was grown aerobically at 30°C in shake flasks in a lactate minimal medium containing L-lactate (0.5% w/v) described previously [7].

Starvation and resuscitation

When the cultures had reached stationary phase (some 10^{10} cells ml^{-1}), agitation was continued at 30°C for up to 3 months. Cultures were then kept aerobically at room temperature without agitation for periods of up to a further 4 months. For resuscitation, cells were inoculated, to a cell density of $2–3 \times 10^8$ ml^{-1}, into fresh lactate minimal medium containing 0.5% (w/v) L-lactate, various concentrations of yeast extract and 0.5 μg ml^{-1} penicillin G, and incubated aerobically at 30°C with agitation. Periodically samples were withdrawn to check total and viable counts.

Most probable number (MPN) method

Serial ten-fold dilutions of starved culture were prepared in test tubes containing autoclaved (121°C for 30 min) supernatant obtained by centrifugation of the suspensions of starved cells. (Other diluents such as lactate minimal medium or phosphate buffer proved unsatisfactory.) Each appropriate dilution was inoculated (0.2 ml inoculum plus 1.8 ml medium) into 10 test tubes containing a medium consisting of a 50:50 mixture of (i) lactate minimal medium with 0.5% L-lactate and 0.05% yeast extract and (ii) supernatant obtained after centrifugation of late logarithmic phase *M. luteus* culture grown in lactate minimal medium. Such supernatants were passed through a 0.22-μm filter (Gelman or Millipore) before use. Test tubes were incubated with agitation at 30°C.

Colony-forming units (cfu) and total cell counts

The viability (cfu) of cell populations was judged by plating on agar plates containing Nutrient Broth E (Lab M) and 1.5% (w/v) purified agar, Lab M No. 1, as described [7]. Similar viabilities were obtained when cells were plated

on lactate minimal medium. Total cell counts were performed on unstained cells using a phase-contrast microscope and a modified Neubauer counting chamber [7].

Chemicals

Nutrient Broth E and yeast extract were obtained from Lab M. L-Lactate and penicillin G were obtained from Sigma. Other chemicals were of analytical grade.

Results and Discussion

Estimation of resuscitable cells by preincubation in the presence of penicillin

In earlier work, we used penicillin (which should kill only actively dividing cells [18,19]) to kill viable cells in populations of starved *M. luteus*, and judged cell death via the decrease in the number of cfu in the culture [1,9]. In the present study, the lysis of starved cells was monitored directly during prolonged cultivation in fresh medium in the presence of penicillin, by carrying out measurements of the total cell counts. The prime purpose of these studies was to maximise the percentage of previously starved cells which could be lysed, since these corresponded to both viable and resuscitable cells under these conditions. The maximum number of cells lysed in such experiments depended rather finely on the penicillin concentration and on the medium composition; in particular, the addition of yeast extract to concentrations much above or below 0.05% resulted in a significant decrease in the percentage of cells that were lysed (data not shown). A similar optimum was also observed in the concentration of nutrients necessary for demonstrating the recovery and growth of ultramicrobacteria [11].

Fig. 1 shows the decrease in the total (microscopic) count of starved cells after incubation with penicillin under these optimal conditions, for cells in three different physiological states. Viable (platable) cells of this strain are very sensitive to the antibiotic, and after 80 h of incubation of a population of fresh cells, lysis had occurred in more than 99.9% of them; by contrast, populations of starved bacteria maintained about 30% of intact cells under these conditions. The onset of lysis for the starved bacteria was also significantly delayed with respect to that for unstarved populations with a high initial viability. A similar delay in the onset of cell resuscitation was found in flow cytometric experiments [1,9]. Since the % viability of the starved cell populations as judged by plating directly on nutrient agar plates was about 0.001% (not shown, but see also [9]), the incubation with penicillin shows that 60–70% of the bacteria in the starved population were at least capable of cell enlargement, and thus perhaps of resuscitation. Control experiments (not shown) indicated that freshly harvested cells incubated in spent growth medium from the stationary phase of a batch culture did not grow and were insensitive to penicillin (i.e. were not lysed by the antibiotic).

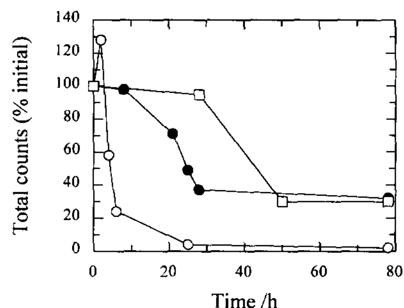

Fig. 1. Effect of time on the total cell count of *M. luteus* cultures incubated with penicillin. Cells were grown and starved as described in Materials and Methods, and were inoculated to a density of $2–4 \times 10^8$ ml^{-1} in lactate minimum medium containing 0.5% (w/v) L-lactate, 0.05% (w/v) yeast extract and 0.5 μg ml^{-1} penicillin G. They were incubated at 30°C with shaking for the times indicated. The inocula, and their initial cfu, were: *M. luteus* cells taken from late logarithmic phase of batch growth (open circles, 8×10^8 ml^{-1}), *M. luteus* cells starved in stationary phase for 3.5 months (closed circles, 2.7×10^4 ml^{-1}), and *M. luteus* cells starved in stationary phase for 6 months (squares, 2.4×10^4 ml^{-1}).

Resuscitation and counting of dormant M. luteus by the MPN method

Although quantitative arguments based on the ratio of 'resuscitable' to total cell numbers, the precision in such measurements, and the kinetics

of resuscitation can be used to counter such charges [9], dormancy studies are always potentially prone to an argument that states that the apparent resuscitation may be due to the genuine growth of a small fraction of the population during the resuscitation phase [13,17]. One way to overcome such difficulties with the presence of small number of viable bacteria in starved populations is to dilute them sufficiently that each tube contains (statistically) less than one potentially colony-forming unit [20,21]. To this end, we sought to use the most probable number (MPN) method [22], which allows one to estimate viable cell numbers by their cultivation in liquid medium at high dilutions. 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 behaviour as judged by direct plating. We therefore carried out numerous MPN experiments in an attempt to demonstrate the growth of starved *M. luteus* at high dilutions (more than 10^5-fold). The concentrations of salts, L-lactate, and yeast extract, and the extent of oxygen saturation of the growth medium, were varied over a wide range, the minimal medium was substituted by a rich one, and cAMP and GTP were tested as additives. In no case could (resuscitation followed by) growth be reliably demonstrated in tubes with dilutions in excess of 10^5-fold when the initial total counts were 10^{10} ml^{-1}. These procedures were shown not to impose a pH stress, by establishing that the pH of early stationary-phase cultures was 7.6 and of 6-month-old cultures was 7.1 whilst the growth media were at pH 7.5.

Earlier, using flow cytometry, we demonstrated the resuscitation of 50% or more of the cells in a population of 3-month-old *M. luteus* cultures which contained a very small number of viable cells [1,9]. However, yet older cultures, with zero (i.e. less than the detection limit of 10^{-7}%) initial culturability under the same conditions, demonstrated only metabolic resuscitation (the appearance of membrane energization as judged by the uptake of rhodamine 123), but such cells were unable to grow. One interpretation of these findings is that a metabolic product of the culturable cells in the population may have promoted the resuscitation of the other, dormant cells. This prompted us to use the supernatant from batch cultures of *M. luteus* grown in lactate minimal medium as a component of the growth medium for the MPN method. In addition, we used the (autoclaved) supernatant from starved cells as the diluent (see Materials and Methods), which we found to provide by far the best resuscitation. Under these conditions, cell growth from inocula taken from suspensions of starved cells at very high dilutions (10^7–10^{10}-fold) was observed after incubation for 72–120 h. Incubation of filtered supernatants, alone or diluted with lactate minimal medium plus 0.05% yeast extract, did not lead to the growth of cells, indicating that very small cells, which might not have been centrifuged out and which might potentially pass through filters with a nominal pore size of 0.22 μm, did not contribute to the growth observed. Fig. 2 shows that for a variety of populations, starved for various times between 2 and 7 months, the MPN counts exceeded the agar plate counts by 10^3–10^5-fold (corresponding to a % viability, i.e. agar plate count/microscopic count, of between 1 and 100%).

From the above, it is evident that some substance(s) which are needed for the resuscitation of dormant *M. luteus* cells can be excreted into the culture medium by viable cells. We have found in preliminary experiments (not shown) that these compounds were apparently heat-sensitive. We also found (not shown) that the addition of supernatant from early stationary phase cultures directly to liquid or solid agar medium did not promote resuscitation. It is evident that the present studies have a strong bearing on the appropriate protocol for the performance of resuscitation experiments, and our findings may well serve to explain some of the data from experiments in which the resuscitation of starved cells at high dilution was unsuccessful [15,17]. In particular, the presence of small numbers of viable cells in a population may actually promote the resuscitation of otherwise dormant cells, rather than necessarily undergoing significant regrowth themselves. As well as the well-known inverse dependence of the length of the lag phase on inoculum size, such findings add to the in-

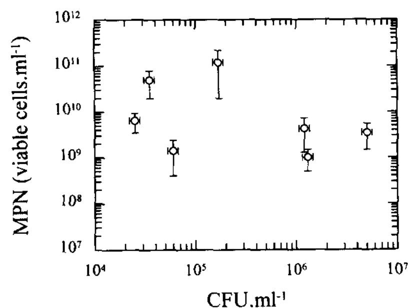

Fig. 2. Relation between viability as judged by MPN and viability judged by plate counts on solid nutrient media of starved cultures of *M. luteus*. Cultures were starved for 2–7 months in spent growth medium as described in the legend to Fig. 1. Viable counts and MPN counts were measured as described in Materials and Methods, the latter in medium containing filtered supernatants from late logarithmic phase cells. Total cell counts, and the ages of the cultures for each point from left to right were as follows: 1.2×10^{10} ml^{-1}, 7 months; 1.2×10^{10} ml^{-1}, 6 months; 2.6×10^{9} ml^{-1}, 2 months; 1.2×10^{10} ml^{-1}, 5 months; 7.2×10^{9} ml^{-1}, 5 months; 1×10^{10} ml^{-1}, 4.5 months; 5.3×10^{9} ml^{-1}, 2 months. The error bars indicate the standard deviations, and the older cells possessed the lower viable counts. The standard deviations come from two sources; in some cases (points 4 and 6 reading from left to right) the entire experiments were done in triplicate. In other cases they are calculated on the basis of the inconsistency between the findings and the combinations given in published tables [31].

creasing evidence from density-dependent transitions (the 'population effect', e.g. [23–27]) that prokaryotes can promote substantial physiological changes via the excretion of signalling molecules [28–30].

In conclusion, two independent approaches in which the (cryptic or net) growth of small numbers of viable bacteria during resuscitation was excluded have served to demonstrate the existence of a high concentration of dormant and hence resuscitable cells of *M. luteus* in populations stored for an extended period in the stationary phase following growth in batch culture. The existence of such dormant cells [1,9] means that if the ability to form a colony is the sole criterion of whether a cell is alive or not, dormancy is probably far more common than death in starving microbial cultures.

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