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A summary of recent work on dormancy in nonsporulating bacteria: Its significance for marine microbiology and biotechnology

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Abstract. We review recent studies from this laboratory on the unusual and interesting biology of starved cells of *Micrococcus luteus*. Cells of the copiotrophic bacterium *Micrococcus luteus* become dormant when subjected to severe nutrient limitation, in that they enter a hypometabolic state in which they are incapable of colony formation (i.e., they are not viable in the usual sense) but from which they may be recovered by resuscitation in a weak nutrient broth (i.e., they were not dead). The results of quantitative measurements exclude the regrowth of initially viable cells as the source of the resuscitated cells. Dormant cells are permeable to normally membrane-impermeant stains, and an early event in resuscitation is the repair of the permeability barrier. When cells are diluted so that no viable cells at all are present in the resuscitation medium, under the conditions of a most-probable-number assay, resuscitation 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. An important reason why microorganisms make secondary metabolites generally is that they are actually pheromones; this has significant implications for the search for bioactive molecules from marine microorganisms.

Dormancy may be defined as a reversible state of low metabolic activity, in which cells can persist for extended periods without division; this often corresponds to a state in which cells are not "alive" in the sense of being able to form a colony when plated on a suitable solid medium, but one in which they are not "dead" in that when conditions are more favorable they may be resuscitated so as to revert to a state of "aliveness" as so defined (Kaprelyants et al. 1993). In natural marine ecosystems, the total cell count obtained microscopically typically exceeds the viable count on nonselective 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.

Attempts to correlate macroscopic properties (such as ATP content, respiratory rate or the extent of lipophilic cation uptake) with the ability or lack of ability to form a colony are doomed to fail (Kell 1988), since if a decline in the level of ATP has occurred, we cannot know whether it is because all the cells have lost half of their ATP or half the cells have lost all of their ATP, or any other combination. Methods of measurement that study individual cells, such as flow cytometry (Kell et al. 1991), are therefore necessary to effect progress.

Dormancy may be correlated with the extent of uptake of rhodamine-123 measured by flow cytometry in individual cells of *Micrococcus luteus* when they are starved in buffer (Kaprelyants and Kell 1992), when grown in chemostat culture at low dilution rates (Kaprelyants and Kell 1992; Davey et al. 1993), and in the stationary phase of batch cultures (Kaprelyants and Kell 1993a; Kaprelyants et al. 1993). Dormant cells are much smaller than viable and resuscitated cells, and reversibly lose the ability to reduce 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Kaprelyants and Kell 1993a,b).

The regrowth of initially viable cells is always a potential artifact in dormancy studies, since the increase in viable counts while the total count is constant within the limit of experimental precision (and before the total count increases via division in the resuscitation medium) is normally much lower than the noise in the latter. Importantly, and for the first time, we were able to exclude this artifact using three quantitative arguments (Kaprelyants and Kell 1993a): (1) resuscitated dormant cells constituted a much higher percentage of the culture than the percentage noise in the total cell count, (2) the rate of resuscitation was far greater than the division rate of the organism, and (3) resuscitation was ac-

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companied by the conversion of small cells into large cells, not merely by the appearance of newly viable cells.

Dormant cells are permeable to normally membrane-impermeant stains, so an early event in resuscitation is the repair of the permeability barrier (Votyakova et al. 1994). This has profound implications for the assessment of cell viability using so-called viability stains.

The previous experiments (Kaprelyants and Kell 1993a) had been done under conditions in which at least a small number of viable cells were initially present. When no such cells were present (Kaprelyants and Kell 1993a) or were removed by dilution (Kaprelyants et al. 1994, Votyakova et al. 1994), resuscitation was not observed. This suggested, as had the kinetics of resuscitation (Kaprelyants et al. 1994; Votyakova et al. 1994), that viable cells may excrete a factor that is required for resuscitation. Indeed, when resuscitation was carried out at high dilution, dormant cells could be resuscitated when supernatant from the early stationary phase of batch-grown cells was added to the resuscitation medium, such that the viable counts (obtained via the most-probable-number method) exceeded the plate counts by three to five orders of magnitude (Kaprelyants et al. 1994). It is possible that the stimulating effect of viable cells, and of supernatant taken from batch cultures, on the resuscitation of dormant cells might be connected in part with overcoming the activity of an antibacterial factor causing self-poisoning of dormant cells during their resuscitation (Mukamolova et al. 1995).

As defined by Stephens (1986), "A pheromone is a chemical excreted by an organism into the environment that acts to elicit a specific response form other organisms of the same species." On this basis, the molecule that *M. luteus* secretes to assist in the resuscitation of dormant forms is indeed a pheromone, and we have recently pointed out (Kell et al. 1995) that the benefits to the producing strain of the production of pheromones of this type in prokaryotes may be accounted for by a simple application of Hamilton's (1964) rule, since organisms in colonies that are mothers and daughters (and so on) have a degree of relatedness close to 1. The existence of such social behavior among prokaryotes is becoming increasingly widely appreciated (e.g., Stephens 1986; Bainton et al. 1992; Kaiser and Losick 1993; Fuqua et al. 1994; Kell at al. 1995; and see, e.g., Raff 1992; Yuen and Gomer 1994 for eukaryotes). Pheromones are by definition excreted, as are those secondary metabolites of pharmaceutical interest; pheromonal activity may therefore serve to explain many of the advantages to be had from secondary metabolite formation generally (e.g., Campbell 1984; Vining 1990; Kell et al. 1995).

In conclusion, we believe that these studies have brought together two important areas of significance to marine microbiology and biotechnology. First, we may state that if the ability to form a colony is the sole criterion of whether a cell is alive or not, it is reasonable that dormancy is likely to be far more common than death in oligotrophic marine (and other) microbial ecosystems and that it may therefore be necessary to add appropriate culture supernatants to effect the resuscitation or recovery of such strains. Similarly, the demonstrable existence of pheromonal behavior among marine prokaryotes such as *Vibrio harveyi* (see Fuqua et al. 1994; Kell at al. 1995) suggests that the screening for bioactive marine molecules might most fruitfully be directed towards those microhabitats in which substantial nutritional shifts are likely.

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