Microbial Diversity and Bioprospecting
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Chapter 10

Resuscitation of "Uncultured" Microorganisms

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> These germs—these bacilli—are transparent bodies. Like glass. Like water. To make them visible you must stain them. Well, my dear Paddy, do what you will, some of them won't stain; they won't take cochineal, they won't take any methylene blue, they won't take gentian violet, they won't take any colouring matter. Consequently, though we know as scientific men that they exist, we cannot see them.
>
> Sir Ralph Bloomfield-Bonington.
> *The Doctor's Dilemma.* George Bernard Shaw.

It is by now well known that the number, and probably the nature (Torsvik et al., 1990a, b, 1996), of microorganisms visible in—and whose activity may often be demonstrated within—a natural environmental or clinical sample is often orders of magnitude greater than the number of cells or propagules that can be isolated and brought into culture therefrom (e.g., Alvarez-Barrientos et al., 2000; Amann et al., 1995; Barer et al., 1993, 1998; Barer and Harwood, 1999; 1998; Biketov et al., 2000; Bogosian and Bourneuf, 2001; Bull et al., 2000; Dobrovol'skaya et al., 2001; Domingue and Woody, 1997; Fredricks and Relman, 1996; Gangadharam, 1995; Gao and Moore, 1996; Head et al., 1998; Kaprelyants et al., 1993, 1996, 1994, 1999; Kaprelyants and Kell, 1993; Kell et al., 1998; Kell and Young, 2000; McDougald et al., 1998; Mukamolova et al., 1995a and 1995b; Postgate, 1976, 1969; Relman, 1999; Rondon et al., 1999; Schut et al., 1997; Smith et al., 2002; Tiedje and Stein, 1999; Votyakova et al., 1994; Watts et al., 1999; Wayne, 1994). What is less than clear, however, is whether these ostensibly "unculturable" cells have permanently lost culturability (i.e., are effectively dead), are killed by (or simply unable to grow on) our standard isolation media, or are in a dormant state from which we might recover them if only we knew how (Barer et al., 1998; Kaprelyants et al., 1993, 1999).

Two important definitions are immediately necessary. First, as noted by Postgate (1976, 1969), we always equate viability and culturability. The consequences of this are at least twofold: (i) phrases such as "viable but not culturable" are to be seen as an oxymoron (Barer et al., 1993, 1998; Barer and Harwood, 1999), and (ii) the definition means that a property such as viability or culturability is not an innate property of a microbe but an operational definition or property. In other words, the (apparent) property of a microbial cell of interest depends not only on the cell itself but on the experimental manipulations we perform to assess its state, as with the Schrödinger's cat paradox (Kell et al., 1998; Primas, 1981). Because we have reviewed this elsewhere at some length recently (Barer et al., 1998; Kell et al., 1998), and it is likely that most or all cells termed viable but not culturable are in fact simply dead or at least irreversibly nonculturable (Bogosian, et al., 2000, 1998; Bogosian and Bourneuf, 2001; Nystrom, 2001; Smith et al., 2002), we do not discuss this specific aspect further. The second definition is that of dormancy, which we define (Kaprelyants et al., 1993) 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 can revert to a state of "aliveness" as so defined. However, the mycobacterial literature refers to a state of latency in which cells also persist for extended periods without net multiplication and possibly without division (Chaisson, 2000; Domingue and Woody, 1997; Flynn and Chan, 2001; Parrish et al., 1998;

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Phyu et al., 1998; van Pinxteren et al., 2000; Wayne, 1994). Such cells may or may not be dormant by the above definition, but there is evidence that they are at least metabolically active (Höner zu Bentrup and Russell, 2001; McKinney et al., 2000). In particular, we note here the important incorporation of the adjective "reversible" in the definition of dormant and dormancy. Finally, we distinguish our use of the phrase "uncultured" organism (when we refer to a microbial strain that can be detected in a natural environment, usually by molecular means, but that is not yet cultured) from "nonculturable" organism (which refers to an organism that has been cultured in a laboratory but that has entered a physiological state in which it is incapable of growth in conditions that normally support its growth).

Because this review is about the resuscitation of uncultured or nonculturable microbes, and such microbes that are successfully resuscitated must by definition have been dormant or latent, we concentrate on this issue, particularly with reference to the actinobacteria that are the source of most of the bioactive secondary metabolites of industrial or applied interest.

LOSS OF CULTURABILITY IN LABORATORY CULTURES—BASIC EXPERIMENTAL ISSUES

Consider the technically undemanding but intellectually rather interesting experiment (taken from Kaprelyants and Kell, 1993) shown in Fig. 1. Part A shows the loss of culturability of *Micrococcus luteus* as a decrease in the plate count at more or less constant total count. On the basis of this type of observation alone, we do not know whether the cells that have lost culturability are dormant or dead. Part B shows what at first sight appears to be the resuscitation of most of these previously unculturable cells (which could not form a colony in a plate count assay) as an increase in culturable (plate) count. However, that fact alone still does not in fact allow one to claim that those ostensibly resuscitating cells were dormant. This is because the noise on the total count (let us charitably say ±10%) is such that the increase in plate count between 32 and 55 h is entirely within the noise of the total (microscopic) count and thus could easily have been due to regrowth (multiplication) of the cells that were already culturable at 22 h; indeed, by the last data point at 58 h most or all of the cells in the culture appear to have initiated regrowth. The clear conclusion is that the presence initially of more than one physiological class of cell confounds the simple analysis based on culturable and total counts alone.

The additional evidence that suggests that the increase in culturable count during the period 32 to 55 h in Fig. 1B is due to resuscitation comes from several sources. First, the kinetics of the increase in culturable count are far more rapid than the known doubling time of *M. luteus* in this medium; the increase in culturable cell count could not be due to growth of the initially viable cells. Second, the fraction of cells that can resuscitate (as seen, for example, at 55 h) before a measurable increase in plate count is about 30% of the total, i.e., significantly greater than the noise in the total cell count. Third, the morphology of the different physiological types of cells allows them to be discriminated, most easily by size; the increase in large cells is exactly matched by the decrease in small

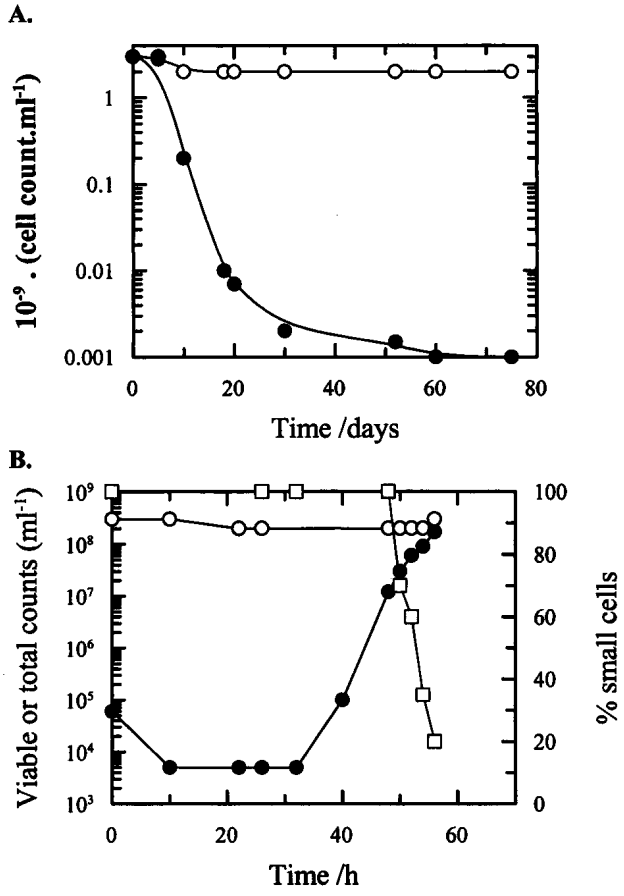

Figure 1. Dormancy and resuscitation in *Micrococcus luteus*. (A) Changes in the viable and total counts of *M. luteus* grown in batch culture and subjected to starvation. Cells were grown and starved, and the total (microscopic) and viable (plate) counts were measured as described by Kaprelyants and Kell (1993). Time zero corresponds to the onset of the stationary phase. (B) Changes in viable and total counts and the percentage of small cells during resuscitation of a starved culture of *M. luteus*. Cells were starved for 75 days, incubated with penicillin G for 10 hours, washed, and resuscitated by the addition of growth medium as described by Kaprelyants and Kell (1993). Total counts, open circles; viable counts, closed circles; percentage of small cells (<0.5 μm in diameter), squares.

cells as the small cells resuscitate (rather than there simply being an increase in the number of large cells).

However, the crucial piece of evidence comes from the use of the most-probable-number (MPN) technique. In the MPN technique, samples of cells are diluted seriatim into a broth that supports their multiplication and whether there is growth is subsequently scored via turbidity. The pattern observed can be compared with the (Poisson) distribution expected from standard tables, and the MPN of cells originally present is then calculated. The advantage of this approach is that the properties of the cells can be determined individually, without the influence of any other cells (or activators or inhibitors) that might be present in the initial medium (Smith et al., 2002). In the system shown in Fig. 1, it was expected that a dormant cell would score as culturable in the MPN assay, as the liquid medium into which the samples were diluted—which was the same as that employed in the previous experiment and identical in each case—should have permitted any dormant cells (ostensibly the vast majority) to resuscitate and then grow. The curious finding, however, was that it did not (Kaprelyants et al., 1994), although the above evidence had already shown that such cells were indeed dormant (i.e., metabolically inactive) (Kaprelyants and Kell, 1993). The big difference in the experiments, however, was that in the experiment of Fig. 1B culturable cells were present during the resuscitation, but in the MPN experiments they were not. This led us to opine that the presence of the culturable cells was necessary not for their own (re)growth but for the production of a substance necessary for the resuscitation of the dormant cells (Kaprelyants et al., 1994; Votyakova et al., 1994). This could be (and was) tested simply by adding sterile supernatant from a culture of viable cells in the MPN experiment. For different cultures, the presence of appropriate concentrations of supernatant increased the culturable count of starved cells in an MPN assay by 3 to 5 orders of magnitude: the supernatant contained a resuscitation-promoting factor (Rpf) produced by culturable cells.

PHEROMONES

Pheromones are substances produced by an organism that have specific effects on other organisms of the same species; although the presence of pheromones in prokaryotes was not widely recognized at the time when the existence of Rpf was proposed (notwithstanding a prescient review by Stephens [1986]), this was a clear example of pheromonal activity. Our own short survey in 1995 (Kell et al., 1995) noted that there was a tendency for molecules such as lactones to be used in gram-negative bacteria whereas gram-positive bacteria often used oligopeptides (often produced from larger precursor proteins). Although there are now many more examples, the basic trend remains unchanged (Fuqua and Greenberg, 1998; Kleerebezem and Quadri, 2001; Kleerebezem et al., 1997; Lazazzera, 2001; Lazazzera and Grossman, 1998).

RPF: A BACTERIAL CYTOKINE FAMILY AND ITS BIOLOGY

Given the resuscitation assay for the *M. luteus* Rpf, we were able to purify it to homogeneity and thus to characterize it (Mukamolova et al., 1998a). The first surprising fact about it was first that it turned out to be a protein of 220 amino acids (Molecular weight of 19,148.5) and, so far as is still known, the protein is not cleaved (apart from the removal of its signal sequence during secretion) to produce activity. In addition, the protein served not only to resuscitate dormant cells but was required for the growth of normal (viable) cells from which it can be removed by washing (Mukamolova et al., 1998a, 1999). This is the activity of a cytokine (Callard and Gearing, 1994; Heath, 1993) or proteinaceous growth factor. The Rpf was extremely potent, this version being active at picomolar concentrations (Mukamolova et al., 1998a, 1999) (and any such estimates are underestimates as not all molecules added will be active; in addition there is a cell-wall binding motif similar to that in lysM [Bateman and Bycroft, 2000] that may sequester it unproductively). Finally, recombinant Rpf cloned in *Escherichia coli* (a host lacking any genes coding for any Rpf homologs and also devoid of any background activity) showed that Rpf alone was the active substance (Kaprelyants et al., 1999; Mukamolova et al., 1998a).

There is a highly conserved Rpf domain (motif) of some 70 amino acids near the N terminus (Kaprelyants et al., 1999; Kell and Young, 2000) of the *M. luteus* Rpf (Fig. 2), and there are by now some 144 known homologs containing this domain (Table 1). The main evidence comes from systematic genome sequencing programs. All examples of proteins containing this domain come from the actinomycetes or actinobacteria, i.e., the high-G+C clade of gram-positive bacteria; indeed we know of no actinomycete that has fewer than one. *M. luteus* seems to have one homolog only (Mukamolova et al., 2002a) (not two as originally suspected [Mukamolova et al., 1998a]), whereas *Mycobacterium tuberculosis* (Cole et al., 1998) and *Streptomyces coelicolor* (Bentley et al., 2002) each have five homologs, one (encoded by

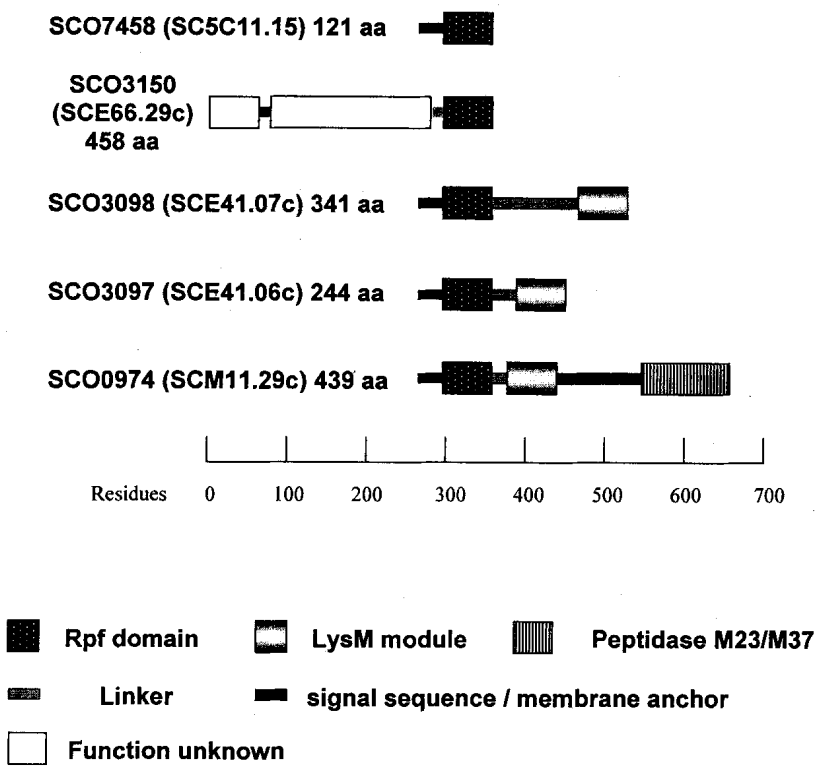

Figure 2. Schematic of the organization of the five Rpf homologs present in the *S. coelicolor* genome.

Rv1009 in *M. tuberculosis*) with a membrane anchor (Fig. 3).

Because the molecule is so potent, it follows (on thermodynamic grounds practically inevitably) (i) that it is the conserved Rpf domain that is responsible for activity and (ii) that there should be at least some level of interspecies cross reactivity. Both predictions are fulfilled (Kaprelyants et al., 1999; Mukamolova et al., 2002b; see also Freeman et al., 2002). In addition, Rpf can increase the culturable count of *M. tuberculosis* obtainable from macrophages ex vivo by several orders of magnitude (Biketov et al., 2000). Similar statements are true for the fast-growing *Rhodococcus rhodochrous* and the slow-growing *M. tuberculosis* in vitro, which adopted a small, coccoid morphology upon lengthy starvation and where cultures appeared to lose completely the ability to multiply on agar plates (Shleeva et al., 2002). All of the above facts make it clear that a basic tenet of microbiology—one cell, one culture—does not hold in its purest form because, in the absence of any cell-wall sequestration of Rpf (which is now obviously seen as potentially beneficial), a cell cannot propagate merely upon being placed in a medium that normally sustains its growth and reproduction (Kaprelyants and Kell, 1996).

Table 1. Some organisms that have been shown to contain Rpf-like genes, with the number of homologs known to date

Organism	No. of genes
Micrococcus luteus	1
Corynebacterium glutamicum	2
C. diphtheriae	3
Mycobacterium tuberculosis	5
M. bovis	5
M. leprae	3
M. avium	4
Streptomyces coelicolor	5
Streptomyces avermitilis	6
Saccharopolyspora erythraea	4

The important biological questions to be asked of Rpf and its homologs include the following:

- Is it necessary for growth and/or resuscitation in vivo as well as in vitro? The answer to this appears to be in the affirmative, at least ex vivo for cells isolated from macrophages (Biketov et al., 2000), although the crucial and difficult truly in vivo experiments (e.g., in tissues obtained from infected hosts [see, e.g., Hernandez-Pando et al., 2000]) have yet to be performed.
- When is it expressed? Expression increases rapidly soon after inoculation of stationary phase cells into fresh growth medium, is maximal during lag and early exponential phase, decreases during late-exponential phase, and ceases in stationary phase (Mukamolova et al.,

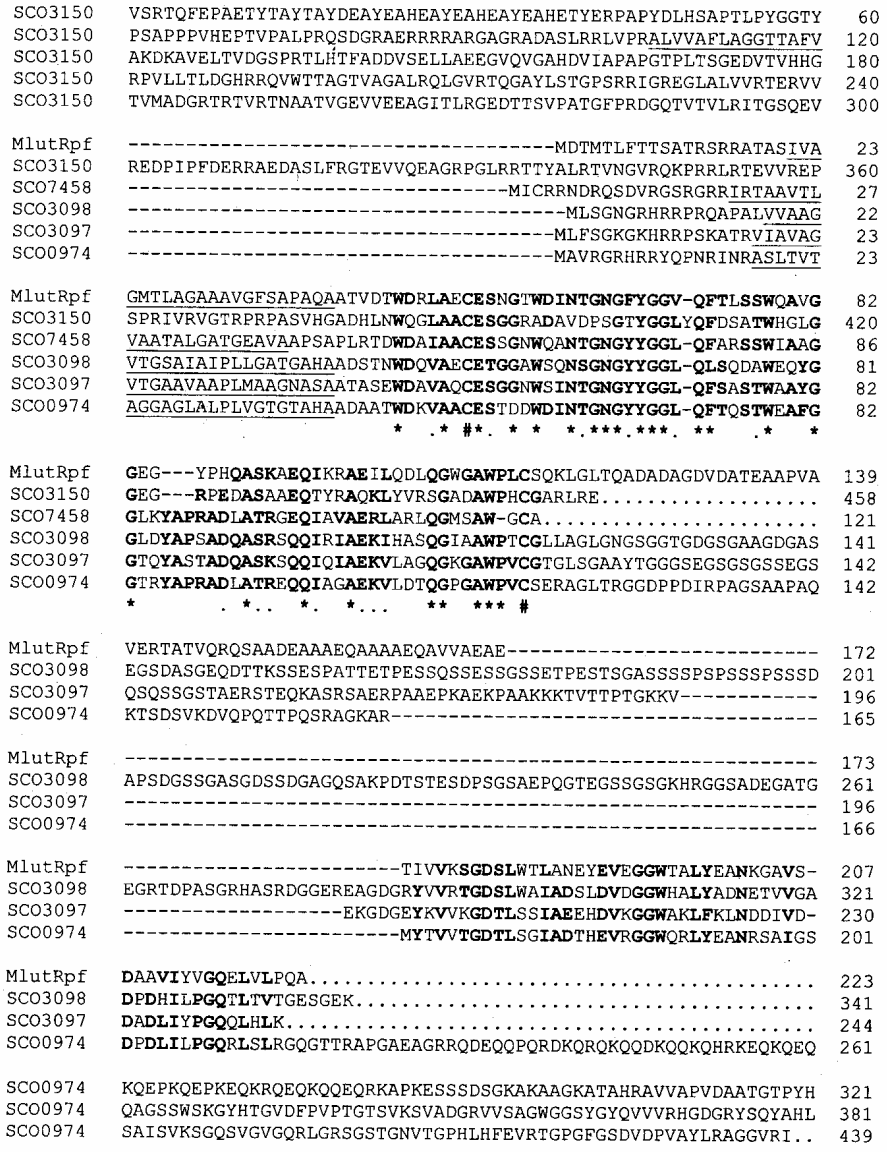

Figure 3. Sequence and alignment of *M. luteus* Rpf with the five Rpf-like gene products found in *S. coelicolor*.

2002a). We do not yet know much about the relative controls on mRNA and protein stability and sequestration that will determine its persistence postproduction.

- Does inhibiting its activity inhibit growth of target actinobacteria? Yes, (polyclonal) antibodies raised against Rpf inhibit its activity, the inhibition being overcome by adding extra Rpf (Mukamolova et al., 2002a).
- What are the mechanisms by which actively growing cells (i) become dormant and (ii) resuscitate from the dormant state? The extent of our ignorance of these matters is well illustrated by an excellent recent review (Barer and Harwood, 1999) in which the authors suggest two possible mechanisms for the transition to dormancy (in *M. luteus*). On the one hand, it might be an ordered developmental program (as is recognized for more obviously developmental processes such as sporulation [Errington, 1996; Losick and Dworkin, 1999] or the stationary phase in gram-negative organisms [Kjelleberg, 1993; Kolter et al., 1993]). On the other, it might simply be a gratuitous and graceful degradation from a state of normal activity and culturability, for which the loss of any number of different functions might be responsible (McDougald et al., 1998). Similarly, the return from dormancy to culturability (resuscitation) would involve either a reproducible and ordered program of gene expression or a more general and stochastic repair and recovery process. Thus far, we have little information about this. We

strongly suspect that dormancy and resuscitation of *M. luteus* are indeed both active and programmed processes. This is suggested by the comparatively coherent timings of the loss and gain of metabolic and biochemical functions (as indicated, for example, by rhodamine 123 uptake) by individual cells in a bacterial population, as observed by flow cytometry (Kaprelyants and Kell, 1993; Mukamolova et al., 1995b).

- Is there a receptor or target for Rpf? Although this is an attractive and almost compelling assumption, nothing is yet known about this. The potency of the bioactivity observed means that possibly only a few molecules per cell are necessary for its activity, and novel methods of detection of binding (e.g., Haupts et al., 2000; Rudiger et al., 2001) may be required.
- What is the actual biochemical role of Rpf? This is as yet unknown. However, so far as the basic phenomenology is concerned, Rpf does have the ability to cause cells that are in what would normally be seen as a nutritionally adequate medium to multiply under conditions in which its absence does not (and which indeed may cause cells to die). In this sense it does indeed exhibit the behavior of a cytokine (Kaprelyants et al., 1999; Kell and Young, 2000; Mukamolova et al., 1998a and b) and as such may contribute to the process of bacterial cell-cycle progression (a topic about which we are also remarkably ignorant in gram-positive bacteria).

RESUSCITABILITY OF BACTERIA TAKEN FROM ENVIRONMENTAL SAMPLES

All of the foregoing leads to the view that it is at least reasonable that the uncultured status of actinobacteria from the environment may be due, at least in part, to the fact that they have indeed become dormant (i.e., only reversibly nonculturable) and thus might be resuscitable given the right nutritional conditions, including the presence of Rpf or an equivalent bioactivity. Certainly what evidence there is shows that most actinobacteria that are culturable will grow on the same general types of media as judged merely by nutritional composition, and in large measure the uncultured actinobacteria observable by molecular means are phylogenetically close enough to cultured clades (McVeigh et al., 1996). The problem, then, is not of culturability but of bringing these strains into culture, where, to paraphrase McLuhan (McLuhan and Fiore, 1971), the medium is the message. Thus, there is evidence that the concentration of nutrients necessary to resuscitate is much lower than that needed for optimal growth (MacDonell and Hood, 1982; Mukamolova et al., 1998b; Shleeva et al., 2002), given that these organisms would normally be considered copiotrophs (Schut et al, 1997). Indeed, the shock of adding specific nutrients to a laboratory culture whose growth was previously limited by such nutrients can cause substrate-accelerated death (see Poindexter, 1987; Postgate, 1967).

AN EVOLUTIONARY CODA

If individual microbes make cell signals that can resuscitate other organisms, as we have shown, this raises a number of evolutionary issues. First, and most obvious, is what is in it for the producer organisms? Genetical kinship theory (Hamilton, 1963, 1964) shows that, provided the benefit of an altruistic action to the recipient times the degree of its genetic relatedness to the producer exceeds the cost to the producer, the action is then selected (Kell et al., 1995), and because the degree of relatedness in clonally propagating bacteria is 1, this selection is likely.

Second, albeit related, the question then arises as to how we might account (in terms of evolutionary selection) for the cross reactivity we have seen for Rpf between organisms from entirely different species or indeed genera, whose degree of kinship is thus negligible. Here the answer is that the competing organisms are not normally associated spatially with the producers, most obviously where we contrast, for example, pathogenic mycobacteria in the lungs of hosts with harmless soil-living streptomycetes, but even at the small-scale level where the existence of very localized microenvironments in the soil is recognized (Bakken and Olsen, 1987).

Another ostensible conundrum relates to how it could make sense to use peptides or proteins as signaling elements under conditions (in the soil or in the sea) in which one would suppose that a large amount of proteolytic activity was present. The probable answer here comes from the potency of the Rpf systems, where the effective concentrations are far below the K_m (Michaelis constant) of known proteases.

Next, although it has been rather commonplace to assume that the humble bacteria are rather homogeneous, especially in axenic cultures in which they are presumed to be genetically identical, phenotypic differentiation (e.g., to produce sentinels [Postgate, 1995] or an insurance policy [Koch, 1987]) is now recognized as widespread (Davey and Kell, 1996;

Sumner and Avery, 2002), and thus we see an important linkage between evolutionary selection and epigenetic phenomena. As to genetic change, perhaps the most interesting recent development in this field, following the original article by Cairns and colleagues (Cairns, et al., 1988), is the recognition that a small fraction of the cells in nongrowing cultures of *E. coli* enter a hypermutable state that can help them escape their condition (Bull et al., 2001; Foster, 1999; McKenzie et al., 2000; Rosenberg, 1997), a phenomenon (the benefits of hypermutation) also observed in chemostats (Riley et al., 2001; Sniegowski et al., 1997), in pathogens in vivo (Oliver et al., 2000), in experimental directed evolution (Zaccolo and Gherardi, 1999), and in evolutionary computation in silico (Corne et al., 2002; Oates et al., 2000).

CONCLUDING REMARKS

It is clear that the vast majority of microbes observable by molecular means have never been cultured. Indeed, a number of biotechnology companies have been started on the premise that, to exploit the biosynthetic potential of such microbes, it will be much easier to express their DNA in other hosts than to try and bring them into culture. Certainly the complexity of natural ecosystems makes their study difficult. However, two facts are pertinent: (i) all known actinobacteria possess genes encoding at least one member of the Rpf family of bacterial cytokines, and (ii) we have demonstrated in the laboratory that such organisms can enter a reversible state of dormancy when starved, and that Rpf can be used to resuscitate them. This makes one optimistic that Rpf—or small molecules mimicking its activity—might be of utility in increasing massively the number of microbes that have been cultured and, thus, the number of useful bioactive substances available to the natural products scientist.

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