INTERCELLULAR SIGNALLING AND THE MULTIPLICATION OF PROKARYOTES: BACTERIAL CYTOKINES.

[1]Arseny S. Kaprelyants, [1]Galina V. Mukamolova, [1]Svetlana S.Kormer, [2]Dieter H. Weichart, [2]Michael Young & [2]Douglas B. Kell

[1]Bakh Institute of Biochemistry, Russian Academy of Sciences, Leninsky pr.33, 117071 Moscow, Russia.

[2]Institute of Biological Sciences, University of Wales, ABERYSTWYTH, Ceredigion SY23 3DD, U.K.

(Received)

*Correspondence to: Dr Arseny S. Kaprelyants, Bakh Institute of Biochemistry, Russian Academy of Sciences, Leninsky pr.33, 117071 Moscow, Russia.

Tel: 00 7 095 954 4047

Fax: 00 7 095 954 2732

Email: ask2@glas.apc.org

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1. INTRODUCTION AND BACKGROUND

Tissue cultures of cells taken from higher, differentiated organisms normally need complex (and mainly polypeptidic or proteinaceous) extracellular growth factors for successful cell division (and even survival (Raff, 1992)). These factors are nowadays usually referred to as cytokines (Callard & Gearing, 1994; Hardie, 1991), and their role is generally understood (cf. (Levine & Prystowsky, 1995)) to involve binding at the cell membrane and the production of second messengers such as cGMP which serve to activate various segments of primary metabolism, which may of course include those responsible for their own synthesis (Alberts *et al*., 1989). Although best known in mammalian systems, such polypeptide growth factors, which can be transported between cells and thus also have the properties of chemical signals, are currently being discovered and identified in higher plants (Matsubayashi & Sakagami, 1996; Matsubayashi *et al*., 1997; van de Sande *et al*., 1996), in multicellular invertebrates (Ottaviani *et al*., 1996), and even in unicellular eukaryotes such as ciliates (Christensen *et al*., 1998; Luporini *et al*., 1995).

Where in evolution such signalling systems may have appeared in a recognisable form is uncertain (Beck & Habicht, 1994; Brown, 1998; Cooper *et al*., 1994; Csaba, 1994; Janssens, 1988; Lenard, 1992; LeRoith *et al*., 1986; Pertseva, 1991; Roth *et al*., 1986), and the apparently conflicting molecular phylogenies (and thus the likely extensive horizontal gene transfer (Koonin & Galperin, 1997; Koonin *et al*., 1997; Doolittle & Logsdon 1998)) recently revealed via comparative genomics suggests that no individual phylogenetic tree is likely to give an unambiguous answer in the short term (Forterre, 1997).

Nevertheless, it is usually assumed in prokaryotic microbiology that each bacterial cell in an axenic culture can multiply independently of other bacteria, providing that appropriate concentrations of substrates, vitamins and trace elements are present in the culture medium, and that implicitly there is

no such requirement for autocrine or paracrine, polypeptide/proteinaceous growth factors. Current laboratory experience seems to be consistent with this, in that the development of bacterial colonies from single cells on agar plates is a commonplace, and the Most Probable Number method is based on the apparently correct assumption that a test tube containing but one viable cell will in due time display visible growth or turbidity. While it is already clear that axenic bacterial cultures do not remotely represent a statistically homogeneous population (Davey & Kell, 1996; Kell *et al.*, 1991; Koch, 1987), these observations tend to be, and are most simply, interpreted as being in favour of "autonomous" growth.

However, such growth is almost always analysed in the presence of culture supernatants (or cell-adherent molecules) that were introduced with the inoculum, and it is at least possible that these inocula may contain potent autocrine or paracrine growth factors, produced by the cells during their previous growth phase, which are in fact necessary for the initiation of regrowth and whose presence would tend not to be recognised in conventional physiological experiments, partly due to the extremely low active concentrations. Indeed, the recently discovered oligopeptide plant hormone ENOD40 modulates cell division in tobacco cell cultures at concentrations as low as 10^{-16} M (van de Sande, *et al.*, 1996), a potency matched only by that of the glycoprotein sex pheromone of the green alga *Volvox carteri* (Hallmann *et al.*, 1998)!

An increasing body of evidence has pointed up the widespread importance of chemically mediated intercellular communications in bacterial cultures for such specific events as sporulation, conjugation, virulence, bioluminescence and so on. Thus, it is now clear that a variety of different autocrine chemical signals (pheromones (Stephens, 1986)), which are produced as secondary metabolites (Bu'lock, 1961; Kell *et al.*, 1995), are responsible for these types of prokaryotic social behaviour, as are exhibited under conditions of obvious cellular differentiation (for review see (Fuqua *et al.*, 1994; Greenberg *et al.*, 1996; Kaiser & Losick, 1993; Kell, *et al.*, 1995; Kleerebezem

et al., 1997; Salmond *et al*., 1995; Swift *et al*., 1994) and many other authors in this volume). Interestingly, while the Gram-negative organisms often use N-acyl homoserine lactone derivatives, the Gram-positives - with the exception of the butanolides of streptomycetes - tend to use proteins and polypetides as their signals (Greenberg, *et al*., 1996; Kell, *et al*., 1995; Kleerebezem, *et al*., 1997). The important properties of such molecules in this context, which discriminates them from nutrients, are that (i) they are produced by the organisms themselves, (ii) they are active at very low concentrations, and (iii) leaving aside the cleavage of prohormones their *metabolism* is not necessary for activity (although they may of course ultimately be degraded).

The question then arises as to whether similar types of signalling may be of significance not only for differentiation but for cell *multiplication* in growing bacterial cultures generally. Our purposes are thus to: (i) develop the idea that this is indeed most probably so, (ii) summarise and bring together the relevant experimental evidence for the involvement of hormones and pheromones in prokaryotic growth and division, and (iii) emphasise the important consequences of this view for a number of apparent (and rather fundamental) microbiological puzzles connected with viability, culturability, dormancy and growth.

2. THE DEVELOPMENT OF BACTERIAL CULTURES DURING THE LAG PHASE

It is a matter of everyday experience that the duration of the lag phase in batch cultures often depends more or less inversely on the size of the inoculum, even when bacterial growth is monitored by counting viable cells ((Penfold, 1914) and see below). (Note that in some studies this “true” inoculum-dependent lag phase is confused with the “apparent” lag when the lag phase is indirectly estimated from uncorrected optical density traces, which must necessarily be “inoculum size dependent” since there is a minimally detectable or threshold change in OD of say 0.01 which equates to some 5.10^6 or 10^7 bacteria.ml^{-1}). The idea that such a dependence could reflect the

accumulation of some growth inducer(s) secreted by cells during the lag phase has long been espoused (see e.g. Hinshelwood, 1946), and indeed the addition of supernatant from log phase bacterial cultures significantly shortened the inoculum-dependent lag phase in a number of cases (Dagley *et al*., 1950; Halman & Mager, 1967; Hinshelwood, 1946; Lankford *et al*., 1966) . However, an inoculum-dependent lag phenomenon may be observable only under a restricted range of conditions: in the case of *Achromobacter delmarvae* an inoculum-dependent lag was detected only in a poor medium but not a rich one (Shida *et al*., 1977). Similarly, the study of inoculum-dependent lags for various *Bacillus* spp. has been performed by using poor medium (Lankford, *et al*., 1966). Dagley and colleagues found that the effect of supernatant on the inoculum-dependent lag itself depends on the size of the inoculum: the largest supernatant effect was observed with the smallest inoculum (Dagley, *et al*., 1950).

Of course *any* substance produced by cells in the culture supernatants may have growth-affecting properties, those of CO_2 being well known (Dixon & Kell, 1989). Notwithstanding the long history of the inoculum-dependent lag phase, however, little is known about the nature of the secreted substances involved in the phenomenon (Kaprelyants & Kell, 1996). Siderophores (formerly "schizokinen") - iron transport compounds - were shown to act as growth factors influencing the inoculum-dependent lag in *Bacillus* cultures (Lankford, *et al*., 1966; Mullis *et al*., 1971), and the activity of such compounds could be mimicked by autoclaved solutions of glucose or other carbohydrates and phosphate (Lankford *et al*., 1957) (although autoclaving glucose separately can of course have quite different physiological effects (Kell & Sonnleitner, 1995)). Batchelor and colleagues reported on a “dramatic reduction” of the lag phase during the regrowth of *Nitrosomonas europea* starved for up to 6 weeks consequent upon the addition of N-(3-oxo-hexanoyl)homoserine lactone (OHHL) to the growth medium (Batchelor *et al*., 1997), a compound known *inter alia* as an autoinducer of luminescence in *Vibrio fisheri* and of antibiotic production in *Erwinia carotovora* (Greenberg, *et al*., 1996; Kell, *et al*., 1995; Swift, *et al*., 1994). It is interesting that there was no lag

phase for starved biofilms composed from the same *N. europea* cells, which strongly supports the density-dependent nature of the recovery process in this case (Batchelor *et al*., 1997), although there are complex interactions between a variety of only partly characterised agonists and antagonists in these types of system (Givskov *et al*., 1996; Kjelleberg *et al*., 1997; Srinivasan *et al*., 1998). Davies and colleagues (Davies *et al*., 1998) found that N-(3-oxodecanoyl)-L-homoserine lactone, a signalling pheromone produced by *Pseudomonas aeruginosa*, was required for the proper development (but not the initiation) of biofilms of this organism, while Bloomquist and colleagues found that there is a significant stimulation of the initiation of streptococcal growth on the tooth surface when the cell concentration reaches a high enough density, and explained this behaviour as the autoinduction of cell growth via cell-to-cell signalling. However the putative signal(s) has not been isolated (Bloomquist *et al*., 1996).

In fact it is relatively straightforward to demonstrate the excretion into bacterial supernatants of compounds which reduce the lag phase in cultures to which the supernatants are added. Figure 1 shows such activity in cultures of starved *E. coli* (DHW & DBK, unpublished observations), and we consider it likely that many other such substances remain to be identified.

3. THE REGROWTH OF BACTERIA FOLLOWING STARVATION, SOME TERMINOLOGICAL QUESTIONS, AND THE PHENOMENA OF LIMITED DIVISIONS.

"When I use a word", Humpty Dumpty said, in a rather scornful tone, " it means just what I choose it to mean, neither more nor less."

"The question is," said Alice, "whether you can make words mean so many different things."

"The question is," said Humpty Dumpty, "which is to be master - that's all."

(Carroll, 1974, orig. 1871)

"*At present one must accept that the death of microbe can only be discovered retrospectively: a population is exposed to a recovery medium, incubated, and those individuals which do not divide to form progeny are taken to be dead........there exist at present no short cuts which would permit assessment of the moment of death: vital staining, optical effects, leakage of indicator substances and so on are not of general validity*" (p.5)

(Postgate, 1976)

"*An organism is considered living or viable if it is capable of continued multiplication; if it is not so capable it is called dead or non-viable.*" (p.31)

(Greenwood *et al*., 1992)

Bacteria commonly face starvation in natural environments, and much information has recently become available concerning the physiological and biochemical changes accompanying bacterial starvation (for review see (Hengge-Aronis, 1996; Kjelleberg, 1993; Kolter *et al*., 1993; Matin, 1994)) which may result in (a) starvation survival (maintenance of cell viability for a prolonged time), (b) cell death, (c) sporulation (for some bacteria) and (d) the formation of resting (dormant) forms of nonsporulating bacteria. Since we wish to avoid the Humpty Dumpty problem (above), we may state that by "dormancy" we mean **a <u>reversible</u> state of low metabolic activity, in which cells can persist for extended periods without division** (Kaprelyants *et al*., 1993). Until recently, dormancy has mainly been connected with bacterial forms which are obviously morphologically specialized, viz. spores and cysts ("constitutive dormancy" (Sussman & Halvorson, 1966)), structures which can be formed by only a limited number of bacterial species. However, there has

recently been much discussion with regard to the possible existence of dormant states of vegetative, nonsporulating bacteria (Kaprelyants, *et al*., 1993).

In particular Roszak and Colwell have proposed (Roszak & Colwell, 1985; Roszak & Colwell, 1987; Roszak *et al*., 1984; Xu *et al*., 1982) that, under some circumstances (mainly starvation), readily culturable bacteria may become nonculturable but retain "viability". Such putative “viable-but-nonculturable” (VBNC or VNC) bacteria (Oliver, 1993) have been proposed to represent some kind of resting (or in some usages active-but-nondividing) bacterial state. However, the term VBNC is an oxymoron because the well-establised convention (Postgate, 1967; Postgate, 1969; Postgate, 1976) is that a bacterial cell should only be considered as viable if it is capable of multiplying (Barer, 1997; Barer *et al*., 1993; Kell *et al*., 1998). In particular, it is necessary to consider that terms such as ‘viability’ can have both a *conceptual* meaning, in which we ascribe a state of say ‘viability’ to the organism *itself*, and an *operational* meaning, in which the state we declare an organism to have is a result only of the outcome of experimental analyses to which we subject it (such that the ‘state’ could in fact vary depending on the experimental outcomes). In the operational realm, such states as ‘viability’ are thus not an intrinsic property of a microbial cell. This crucial distinction, though perhaps unfortunately unfamiliar to many microbiologists, is well known both in the microscopic world of quantum physics (Primas, 1981), especially as the Schrödinger’s Cat paradox (Kell, *et al*., 1998), and in the macroworld of human existence in which ‘death’ can be ascribed only *a posteriori* (Watson, 1987). *Only the operational definitions are free of paradoxes and difficulties*.

Notwithstanding that many of the problems accompanying such studies are semantic, we can at least say that if this putative “VBNC” state of cells exists, it should be reversible if it is to be accepted as a specialised form of the bacterial life cycle (and these ostensibly non-culturable cells are not therefore simply dead). While many attemps to find conditions for the recovery (“resuscitation”) of cells from so-called “VBNC” states have been made, almost all published studies up to now unfortunately fail

to discriminate adequately between resuscitation/recovery and the regrowth of any viable (culturable) cells initially present in the “VBNC” population; only a few examples can be considered to have shown true resuscitation (as defined by a return to culturability) of cells under conditions in which the contribution of ‘initially-viable’ cells is excluded (Bogosian *et al*., 1998; Kell, *et al*., 1998).

Little is known about the processes that occur during the outgrowth of starved, “VBNC” or ultramicrobacterial (Morita, 1988) cells, and where information is to hand the evidence suggests a profound differentiation in terms of the culture's ability to emerge smoothly from the lag phase. In addition, the problem of culture heterogeneity (the coexistence of viable, injured, dormant and dead cells) greatly complicates studies of this process. Nevertheless some peculiarities of cell behaviour during the “outgrowth” phase could be of interest in the light of the problems discussed in this review.

Thus, it was found that a fraction of the cells in a population of starved marine bacteria ceased growth after one or a few cell divisions when placed on an agar surface; cells within such "microcolonies" were morphologically different from cells which produced visible macrocolonies (Torrella & Morita, 1981). *Pseudomonas fluorescens* cells starved in soil for 40 days were tested for their ability to grow in different fresh media; an epifluorescence technique showed that within the first 40 hours of incubation cells divided only 2-3 times, after which cell division stopped (Binnerup *et al*., 1993). A similar result was obtained when soil bacteria were subjected to cultivation on nutrient-poor media (Winding *et al*., 1994).

We found that *Micrococcus luteus* cells starved for 3-6 months in a prolonged stationary phase consist of 10-90% of dormant forms which could multiply several times (up to 10-17) when resuscitated in fresh liquid medium (which allows *normally viable* bacteria to grow rapidly to a high

optical density). After this the cell growth stopped, with final cell concentrations of no more than 10^6 cells.ml^{-1} (Mukamolova *et al*., 1995), although the cells remained metabolically active as judged by flow cytometry (Votyakova *et al*., 1994). A variety of cognate studies are reviewed elsewhere (Kaprelyants and Kell, 1996).

It is reasonable that the phenomenon of "limited divisions" is actually rather common in microbiological practice, but because the threshold for optical detection of growth typically lies between 10^6 and 10^7.ml^{-1} it has not been registered in many cases (and we know of many unpublished or anecdotal cases in which it is known that such culture growth is detectable only with 'large' inocula). What kind of mechanism(s) may be responsible for this phenomenon?

Recently the view has been expressed that the inability of some starved bacteria to grow under conditions which normally support their growth is due to imbalanced metabolism during the start of cell regrowth, as a result of which the accumulation of free radicals leads to "self-destruction" by the cells (a suicide response) (Bloomfield *et al*., 1998), and it is true that some recovery experiments show that concentrations of nutrients which normally supported bacterial growth are too high for the recovery of starved cells, e.g. with *Vibrio* (MacDonell & Hood, 1982) or *M. luteus* (Mukamolova *et al*., 1998b). The significance of aeration for the further resuscitation of dormant *M. luteus* has also been appreciated (Mukamolova, *et al*., 1998b), while it is interesting that embryos of *Artemia franciscana* can persist in a dormant state for several years when fully hydrated but under strong anoxia (Clegg, 1997). But by definition any such "suicide response" cannot for instance properly explain the phenomenon of "limited divisions" in which starved or dormant cells during resuscitation can make several divisions before growth ceases, nor indeed resuscitation generally since (*pace* (Bloomfield, *et al*., 1998)) dormancy is *reversible* but suicide is not (Barer *et al*., 1998; Kell, *et al*., 1998).

Other mechanisms that may be responsible for causing the cessation of cell growth in the above circumstances include the accumulation of inhibitory substances during the growth of previously starved cells. In this context, we demonstrated the secretion of a "killer factor" during the resuscitation of starved *M. luteus* cultures, which inhibited the growth of viable bacteria (Mukamolova, *et al*., 1995), while MacDonell and Hood (MacDonell and Hood, 1982) described a recovery method for bacteria from estuarine waters, which included several passages of cells from plate to plate until they became able to produce visible colonies, consistent with the removal of an inhibitory substance. Possibly, these two mechanisms are linked and accumulated inhibitors are products of cell destruction (by whatever mechanism).

We showed in experiments with dormant *M. luteus* that the presence of a small fraction of viable cells at the onset of resuscitation facilitated the recovery of the majority of the remaining (dormant) cells. The cell density-dependence of the kinetics, or population effect, would suggest that this is due to the excretion of some factor(s) which promoted the transition of cells from a state incapable of growth and division to a colony-forming state (Votyakova, *et al*., 1994). In subsequent experiments, the addition of supernatants from **growing** *M. luteus* cultures to the starved culture relieved the inability of the cells to divide for more than a limited number of times, and allowed the resuscitation of cells to normal, colony-forming cells (Kaprelyants *et al*., 1994) (see Fig 2). Possibly in this (and probably in other similar) cases starved cells have a lowered ability, at the beginning of cell growth, to excrete an appropriate growth factor(s) needed for the stimulation of cell division. As a result only some of the cells in a population can multiply, while the gradual accumulation of poisoning substances formed by nondividing cells leads to a decreased excretion of the factor and eventually to the cessation of cell multiplication. More generally, there may be a limited quota of growth factor whose steady-state concentration is low in poor media and which is degrading over time, and being shared out between cells over generations until its concentration *per cell* is inadequate for division.

But overall, the main conclusion which can be made from this section is that, whilst in many cases starved cells become more resistant to environmental insults (Hengge-Aronis, 1996; Kjelleberg, 1993; Matin, 1991), starvation may be accompanied by an increased sensitivity of cells to specific chemical inducers or inhibitors of cell division which is not observable under normal growth conditions.

4."MICROENDOCRINOLOGY"

Clearly the communication between bacterial cells in an axenic population ("quorum sensing") resembles the well known chemically-mediated interactions between cells in higher organisms, as mentioned above, and it seems logical to ask whether the "bacterial" chemical language of communication might contain some homologous "words" (molecules) or "grammar" (modes of action and regulation) as that of higher organisms? Indeed, a number of studies have revealed the presence of vertebrate hormone-like substances in bacteria, which were specifically active on mammalian cells. The list includes steroid and polypeptide hormones (including insulin); moreover, specific, high-affinity binding proteins for many mammalian hormones were found in bacterial cells (for review see (Lenard, 1992; LeRoith, *et al*., 1986)). In a most interesting study, an autocrine growth stimulation function of chorionic gonadotropin-like protein from *Xanthomonas maltophilia* has been associated with a fully sequenced 48-kDa membrane-bound protein (Grover *et al*., 1995).

Lyte (Lyte, 1992) has developed the idea that the growth and virulence of pathogens *in vivo* depends strongly on host-derived hormonal signals, and it is certainly known that some hormones can stimulate the growth of microorganisms; examples include catecholamines (in Gram-negative bacteria) (Lyte *et al*., 1997; Lyte *et al*., 1996; Lyte & Bailey, 1997; Lyte & Ernst, 1992), serotonin (*S. faecalis*) (Strakhovskaya *et al*., 1993) and insulin (*Neurospora crassa*) (McKenzie *et al*., 1988), while inhibition of growth has been observed for insulin in *Pseudomonas pseudomallei* cultures

(Woods *et al*., 1993). The stimulation of the growth of virulent strains of *E. coli* by interleukin-1 (Denis *et al*., 1991; Porat *et al*., 1991) and by granulocyte-macrophage colony-stimulating factor (Denis *et al*., 1991) was reported in 1991. Later the same effect was described for interleukin-6 on virulent *Mycobacterium avium* (Denis, 1992) and for transforming growth factor-β-1 which enhanced the intracellular growth of *M. tuberculosis* in monocytes (Hirsch *et al*., 1994). A significant acceleration of growth of *Mycobacterium avium, M. tuberculosis* but not *M. smegmatis* was observed when they were cultured in the presence of 5-500 $ng.ml^{-1}$ epidermal growth factor (EGF), and a specific receptor for EGF was identified as a glyceraldehyde-3-phosphate dehydrogenase (Bermudez *et al*., 1996) which has been shown to bind plasmin and some other proteins on the surface of streptococci (Pancholi & Fischetti, 1992). It is worth noting that when the activity of the appropriate hormone or cytokine was tested in all these studies, the effect on bacterial growth *rate* (usually in an exponential phase) was not at all pronounced (excluding the last case with EGF). This is not very surprising, as competition experiments are far more sensitive in revealing small changes in growth rate or fitness (Baganz *et al*., 1997; Dykhuizen, 1993; Dykhuizen & Hartl, 1983; Thatcher *et al*., 1998), yet this problem was responsible for the apparently difficuties in reproducing some of these results (see (Kim & Le, 1992; Porat *et al*., 1992)).

It important to note that in most of these experiments the conditions were actually the least suitable for detecting a stimulatory effect, viz. the use of rich medium, a relatively large inoculum and the assessment of the effect during the log phase. Lag-phase studies (see above) indicate that the use of large inocula (with their attendant carry-over of stationary-phase supernatant) and rich medium can mask the stimulatory effect of growth factors. Indeed, Lyte and Ernst found that catecholamines had a detectable effect on bacterial growth only if the starting concentration of cells was low (Lyte and Ernst, 1992). Table 1 summarises cases in which either true pheromonal (autocrine and/or paracrine) effects on microbial growth have been observed or in which hormones usually considered characteristic of higher eukaryotes have been shown directly to stimulate microbial growth.

5. CYTOKINES OF UNICELLULAR EUKARYOTIC ORGANISMS AND CULTIVATED NUCLEATED CELLS

From the physiological point of view, the growth and development of cultures of bacteria and of nucleated unicellular organisms *in vitro* exhibit many similarities, and it could be expected that the mechanisms of control of cell multiplication - which might include the participation of cytokines - are also similar or even identical for the two kinds of cells. Indeed, the need for factors synthesised by cells for their further multiplication has been noted for unicellular organisms such as ciliates. If cells of *Tetrahymena thermophila* were inoculated into a poor medium at a concentration of <750 cells per ml they could not grow at all until either supernatant from growing culture or various "inducers" including lipids, alcohols, insulin, hemin and porphyrins were added (Christensen, *et al*., 1998; Christensen *et al*., 1995; Wheatley *et al*., 1993). These authors pointed out the ability of *Tetrahymena* cells to produce “insulin-related” material and speculated that insulin-like autocrine factors might be involved in the phenomenon (in the *Tetrahymena* model insulin is active at concentrations $<10^{-15}$ M) (Christensen, *et al*., 1995). At the same time it was found that the proliferation of *Tetrahymena periformis* was stimulated by α2-macroglobulin, a 120 kDa protein found in foetal calf serum, and specific antibodies to this protein detected a 180kDa component in *Terahymena* extract (Hosoya *et al*., 1995). The "growth factor" for another ciliate, *Paramecium tetraurelia*, has been isolated from culture medium as a 17 kD protein (Tanabe *et al*., 1990), but remains regrettably unsequenced. It is interesting that crude samples of spent medium of *Terahymena* were active as growth factors for *Paramecium* cells (Tokusumi *et al*., 1996), and the possible relationship between *Paramecium* and *Tetrahymena* cytokines remains to be established.

Cultivation of *S. cerevisiae* in a very poor medium from a small inoculum was not possible until glucose (0.001%) or the nonmetabolizable 6-deoxy-glucose were added to the culture medium, and it was suggested that glucose here served as a signalling molecule for proliferation; some tetrapyrroles (which have a role in the regulation of cAMP levels) had a similar effect (Overgaard *et al.*, 1995).

The cytokine pheromones of the ciliate *Euplotes raikovi* seem to be the presently best characterisied among unicellular species. These secreted pheromones are represented by several peptides (M_r about 4-5kDa) which had traditionally been associated only with the organisms' mating activity. However it was found that they also possess growth-stimulating activity (Vallesi *et al.*, 1995). These cytokines are secreted in precursor forms which undergo two proteolytic cleavages during exocytosis (Luporini *et al.*, 1994). Moreover, by an alternative splicing mechanism the cell secretes each pheromone in two forms: a membrane-bound isoform (M_w 14kDa) and soluble peptide. The first form has an extracellular (C-terminal) domain with an amino acid sequence identical to that of the soluble form (Luporini, *et al.*, 1994). Unusually, this membrane-bound form serves as a receptor for soluble pheromone, and analysis of the crystal structure of the soluble forms revealed extensive helix-helix interactions between adjacent molecules which may mimic cytokine-receptor interaction (Weiss *et al.*, 1995), since the binding of effector to the receptor results in clustering of proteins (with subsequent activation of signal transduction pathways) and formation of tetrameric complexes on the cell surface (Vallesi, *et al.*, 1995). In addition to a receptor function in the membrane the membrane-bound form may play role as a so-called juxtacrine growth factor by interacting with a receptor on the surface of a neighbouring cell. This type of ligand-receptor interaction has been proposed for vertabrate cytokines belonging to the epidermal growth factor (EGF) family and some other cytokines (colony-stimulating factor, tumour necrosis factor and others). It was suggested that "juxtacrine" stimulation may play a role in the communication between closely adjacent cells, for

example for more precise targeting of growth factor in developing tissue when freely diffusing factors are less appropriate (Massague & Pandiella, 1993).

These similarities between the growth factors from ciliates and vertebrates allows interesting speculations regarding the evolutionary development of mechanisms of cell proliferation control (Luporini, *et al*., 1994), based on the fact that human interleukin IL-2 is a very active competitor for the binding of the Er-1 ciliate cytokine to its receptor. While these two signalling molecules are quite different in their overall composition, nevertheless one (short) conserved segment does reveal significant sequence similarity (Luporini, *et al*., 1994).

Similarities also occur between bacterial cells and cultivated cells of higher organisms. Thus, cultivation of pre-starved fibroblasts resulted in a low cell concentration at the end of cultivation in normal medium, irrespective of seeding densities (Pignolo *et al*., 1994). Lens cells held in cultures with a low initial concentration of the cells die off rapidly, while conditioned medium from high-density cultures promoted their survival (Ishizaki *et al*., 1993). It has been proposed that there is a universal mechanism for cell death, as in these cases, based on the idea that there may have been an absence or insufficient secretion of one or more factors which prevent normally growing cells from initiating apoptotic death (Raff, 1992). A similar mechanism probably works in *Tetrahymena* (Christensen, *et al*., 1995).

Thus autocrine polypeptide substances can help effect cellular growth by at least two general mechanisms: by stimulating growth *per se* and/or by inhibiting processes leading to stasis or to death.

6. ISOLATION AND PURIFICATION OF RESUSCITATION PROMOTING FACTOR (Rpf) FROM *M. LUTEUS*; THE FIRST BACTERIAL CYTOKINE

Growing *M. luteus* cells secrete a resuscitation-promoting factor (Rpf) which (a) promotes the resuscitation of dormant cells and (b) reduces the apparent lag phase of cultures of the same organism when inoculated at low density (see above and (Kaprelyants, *et al*., 1993; Kaprelyants & Kell, 1992; Kaprelyants & Kell, 1993; Kaprelyants and Kell, 1996; Kaprelyants, *et al*., 1994; Kell, *et al*., 1995; Mukamolova, *et al*., 1995; Mukamolova, *et al*., 1998a,b; Votyakova, *et al*., 1994)). Rpf was heat-labile, non-dialysable and trypsin-sensitive and it was purified to homogeneity from culture supernatants (for details see (Mukamolova *et al*., 1998a)). The resuscitation and apparent lag phase-reducing activity corresponded to a protein with an apparent MW of 16-19 kDal as estimated by electrophoresis and gel filtration. Picomolar concentrations of Rpf increased the number of culturable *M. luteus* cells from dormant populations by several orders of magnitude and they also stimulated the growth of *M. luteus* in batch culture in a lactate minimal medium. In view of the low concentrations necessary for activity, a trivial nutritional role for (proteolytic degradation products of) Rpf was discounted. Moreover, Rpf was also active in the presence of yeast extract (0.05%) and in rich medium (Broth E) (unpublished). Rpf therefore has the properties of a bacterial cytokine.

The gene encoding Rpf was isolated from *M. luteus* and sequenced (Mukamolova, *et al*., 1998a). It encodes a 220 amino acid product with a 38-residue signal sequence (Fig. 3). The predicted size of the secreted form of the gene product is 19,148 Dal, and its predicted amino acid sequence agrees with protein microsequence data obtained from RPF purified from culture supernatants. To confirm cytokine activity, a histidine-tagged version of the secreted form of Rpf (i.e. lacking the signal sequence) was expressed in *E. coli* and purified to homogeneity by Ni^{2+} chelation chromatography. The recombinant Rpf reduced the apparent lag phase of viable cells of *M. luteus* at picomolar concentrations (Fig.4) just as did Rpf isolated from *M. luteus* culture supernatants (Mukamolova, *et al*., 1998a).

Genes similar to *rpf* appear to be widely distributed among the high genomic G+C cohort of Gram-positive bacteria. Database searching has revealed that similar genes are present in *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Fig. 3). Southern hybridisation and/or PCR experiments with *rpf*-specific primers have revealed that a second similar gene is present in *M. luteus* and that similar genes are also detectable in several other organisms including *Mycobacterium smegmatis*, *Mycobacterium bovis* (BCG), *Corynebacterium glutamicum, Streptomyces coelicolor* and *Streptomyces rimosus*. On the other hand, *rpf*-like genes are not present in any of the other organisms whose genomes have been sequenced to date (*Aquifex aeolicus*, *Archaeoglobus fulvidus*, *Bacillus subtilis*, *Borrelia burgdorferi, Escherichia coli, Haemophilus influenzae*, *Helicobacter pylori, Methanococcus jannaschii*, *Methanococcus thermoautotrophicum*, *Mycoplasma genitalium, Mycoplasma pneumoniae*, *Saccharomyces cerevisiae, Staphylococcus aureus, Streptococcus pneumoniae, Synechocystis* PCC6803).

The N-terminal region of the secreted Rpf of *M. luteus* is substantially similar (42% of residues are identical or are conservatively substituted over a 69-residue segment) to the predicted products of several genes in *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Fig. 3). The former organism encodes five Rpf-like gene products, according to the completed genome sequence (http://www.sanger.ac.uk/Projects/M_tuberculosis/). If we assume that these structurally similar mycobacterial proteins perform a similar biological function to that of the *M. luteus* Rpf (evidence to support this assumption is presented below), the following hypothesis can be proposed. One of the Rpf-like gene products (g2052146) is much larger than the others and is predicted to have a membrane anchor at its N-terminus. The Rpf-like domain lies at the extreme C-terminus of the protein, and it is tempting to speculate that this gene product may traverse the bacterial cell wall to present its Rpf-like domain at the cell surface functioning as a signalling molecule over the short distance that separates adherent cells (juxtacrine function). Indeed, the formation of cell aggregates during growth of *M. tuberculosis* is well known . All the other known Rpf-like gene products

(including four in *M. tuberculosis*) are secreted (Fig. 3). There is also an evident and intriguing consonance between the Rpf-like gene products of *M. tuberculosis* and the *Euplotes raikovi* cytokine, where the membrane-bound form serves as a receptor for the soluble pheromone.

Given the significant similarity between *M. luteus* Rpf and the products of other genes found in several Gram-positive bacteria, we tested whether purified Rpf can also be employed as a growth factor for mycobacteria. It is active with both rapidly growing and slowly growing species. Growth of *Mycobacterium bovis* (BCG), *M. tuberculosis*, *Mycobacterium avium* and *Mycobacterium kansasii* in Sauton medium was stimulated by RPF. Similar results were obtained with *M. smegmatis* growing in either a minimal or a rich medium (Mukamolova, *et al*., 1998a). Growth of *M. bovis* (BCG) in Sauton medium was also strongly stimulated by Rpf; growth occurred after 14 days, whereas the control lacking Rpf showed no visible growth after 20 days (Fig 5). Additional experiments showing that Rpf stimulates the growth of *M. tuberculosis*, *M. avium*, *M. bovis* (BCG) and *M. kansasii* are summarised in Table 2.

These results may have important implications for (a) the detection of mycobacteria in clinical samples and (b) controlling the progression of mycobacterial infections.

7. WHEN ONE VIABLE BACTERIAL CELL CANNOT MULTIPLY IN WHAT SEEM NORMALLY APPROPRIATE CONDITIONS; THE DEPENDENCE OF GROWTH ON RPF

In suggesting a crucial role of secreted bacterial cytokines for cell multiplication we may expect that there are conditions in which cell growth would be arrested (or significantly retarded) without externally added cytokine. Clearly, such conditions may be expected to include sub-optimal media, a small inoculum size, cellular depletion of endogeneous cytokine, or any combination of these.

Similarly, any treatment resulting in the degradation or decreased production of Rpf by cells should influence their growth pattern. Indeed, whereas unwashed *M. luteus* cells proliferate normally in liquid lactate minimal medium (LMM) (Kaprelyants and Kell, 1992), the proliferation of a small inoculum of washed cells in this medium appeared to be absolutely dependent on added Rpf over the 160 h duration of the experiment (Mukamolova, *et al*., 1998a).

Additional information on the necessity of Rpf for bacterial growth has been obtained from growth experiments employing conditions even less favourable than LMM. To this end succinate was used instead of lactate in minimal medium with the results shown in Table 3 and Fig 6. Succinate minimal medium (SMM) does not normally support macroscopically observable *M. luteus* growth when the inoculum is less than 10^5 cells ml^{-1} (cells underwent only a few divisions after which growth stopped; this might be due to carry-over of some Rpf with the unwashed inoculum). However, addition of purified Rpf (4 ng ml^{-1}) resulted in sufficient cell growth to form a turbid suspension (Fig 6). It should be stressed that washed cells have not lost the ability to make colonies on LMM agar, and normal cells can also grow on agar prepared with SMM without added Rpf. This may be due to the fact that neighbouring cells are in intimate contact during colony development, which should facilitate cell-cell communication by locally accumulated Rpf, as well as juxtacrine signalling by cell surface-associated proteins.

M. luteus cells taken from an extended stationary phase grow very poorly in LMM. The culturability of such cells was checked by two methods: (1) plating out on agar plates and (2) MPN assay. Table 3 shows significant differences between the MPN count and cfu for cells incubated for 100h in stationary phase (the latter was almost identical for either LMM or rich medium plates (BrothE)). The under-estimation of viable cells by MPN indicates that at least 1000 viable cells (as judged by cfu) must be present per tube if they are to produce visible growth. The addition of Rpf to the MPN dilutions led to almost identical numbers of viable cells as estimated by the two methods,

demonstrating the ability of one single stationary phase cell to grow in a tube provided Rpf was present (Table 3). A similar effect was obtained when MPN assays were performed either in SMM using untreated cells or in LMM using cells that had been washed several times (Table 3). In both cases the underestimation of viable cells by the MPN method (in comparison with viable counts judged on plates) demonstrated that at least 10^5 "succinate" cells and about 10^2 "washed" cells are required per tube for visible growth. Again, the addition of Rpf resulted in very similar viable counts as judged by the two methods. Similar effects have been observed with other organisms. For example, cultures of *B. subtilis* in poor medium inoculated with fewer than 100 cells per flask failed to initiate growth unless "shizokinen" were added (Lankford, *et al*., 1966) . Cells of *Pasteurella* (now *Francisella*) *tularensis* did not grow to a high optical density from an inoculum of $< 10^5$ cells per flask, unless supernatant taken from a growing culture of the same organism was added (Halman *et al*., 1967).

These experiments clearly show (i) the dependence of bacterial growth on secreted growth factors or cytokines and (ii) the benefit of studying "unfavourable" conditions to make this dependence most visible (Christensen, *et al*., 1998; Wheatley, *et al*., 1993). How comparable these phenomena are in other organisms remains to be established, but it is clear that the basic "one cell-one culture" principle of microbiology may not apply in some circumstances.

The mechanism(s) responsible for "nonculturability" (in the operational sense) of bacterial cells depleted of exogenous Rpf in the above experiments is/are not yet clear. For such cells to commence multiplication Rpf must accumulate to a sufficient concentration. The time required will depend on both the initial cell density and the metabolic activity of the cells. A metabolically active cell may have a finite "life time" during which it can survive without division. If held in stationary phase for a period exceeding this life time, cell death ensues by a mechanism(s) which remains to be elucidated.

More generally, and as is well established in eukaryotic cells (Raff, 1992), programmed cell death might play a role in bacterial cell auto-degradation. To date "bacterial apoptosis" (Hochman, 1997) has been described as a mechanism for maintaining plasmids in bacteria (e.g. the "addiction module" in *E. coli* extrachromosomal elements (Yarmolinsky, 1995)), a similar "module" consisting of two genes *mazE* and *mazF* was recently found in the chromosome of *E. coli* (Aizenman *et al*., 1996). MazF is a stable protein which is toxic for cell while MazE is a less stable protein which protects the cell from MazF. Under starvation conditions the level of ppGpp increases and inhibits the expression of both genes, which results in a toxic effect of MazF and cell death (Aizenman, *et al*., 1996). This mechanism works under extreme conditions of deep starvation. However it might also be responsible for the initiation of cell lysis under conditions insufficient for the secretion of growth factor(s), as with the initiation of apoptosis in eukaryotic cells (Raff, 1992).

8. POSSIBLE SIGNIFICANCE FOR BACTERIAL PATHOGENICITY - NOVEL TARGETS, NEW VACCINES AND DRUGS.

The self-promoting mode of bacterial cell growth can have significant implications for medicine and epidemiology. This follows from several phenomena, the most evident of which is an apparent "nonculturability" of some pathogenic bacteria. Indeed, there are several unresolved public health problems potentially involving transition to and from a "nonculturable" state of an infective agent. Principally, these concern aspects of the epidemiology and natural history of infective diseases which cannot be reconciled with the sample pattern from which the known causal organisms can be isolated. 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 might be accounted for on the basis of their being present in a reversibly nonculturable state. For both these organisms, environmental investigations have provided evidence for the presence of "nonculturable" cells in appropriate samples (Brayton, 1987; Pearson, 1993)

while *in vitro* studies have demonstrated their capacity to form metabolically active cells which could not be grown immediately (Rollins & Colwell, 1986; Xu, *et al*., 1982). The list of organisms for which similar phenomena have been claimed (albeit less extensively) is substantial (Kell, *et al*., 1998; Oliver, 1993). From the above discussion it is clear that the involvement of bacterial growth factors in recovery from starvation or resuscitation from dormancy should significantly change the currently accepted methodology for monitoring the environment for biological hazards, and there is evidently a necessity to formulate new protocols for the isolation of bacteria from natural samples, including cultivation of purportedly "nonculturable" forms in liquid media supplemented by appropriate growth factors.

Further medically significant areas where transition to and from putative "nonculturable" states have potential relevance include bacterial infections which have a clinically dormant or latent phase and the effects of antibiotics (Domingue & Woody, 1997). Tuberculosis (Gangadharam, 1995; Parrish *et al*., 1998; Wayne, 1994; Young & Duncan, 1995) and melioidosis (Dance, 1991) provide examples of the former. This has especially been suggested for *M. tuberculosis*, which are capable of adapting to dormancy in the tissues of humans and experimental animals, leading to latency of the disease; this is strongly supported by the transition of viable *M. tuberculosis* cells to nonreplicating cells under microaerophilic conditions (Wayne, 1994). Domingue and colleagues demonstrated the presence of bacterial 16S rRNA genes characteristic of Gram-negative bacteria in biopsies of patients with interstitial cystitis, although routine cultures of bacteria from patients' urine were negative. It was suggested that a persistence of such bacteria in a dormant form is involved in the etiology of this disease (Domingue, 1995; Domingue *et al*., 1995). In this connection it is worth mentioning the debates on the role in the bacterial persistence of so-called coccoid forms of some pathogenic bacteria. This forms have been described for *Campylobacter jejuni* (Beumer *et al*., 1992), *Helicobacter pylori* (Cellini *et al*., 1994), *Mycobacterium tuberculosis* (Khomenko & Golyshevskaya , 1984) and unknown bacteria in patients with cystitis (Domingue, *et al*., 1995) as a

result of either prolonged starvation *in vitro*, or persistence in patients *in vivo*. These forms represent a “nonculturable” state, and it has been suggested they might be infective agents representing dormant forms (Domingue and Woody, 1997). Whilst the experimental evidence for their dormancy and resuscitation to viable bacteria is largely not yet to hand (Kell, *et al*., 1998), reversibly “nonculturable” or dormant cells of pathogens could provide a straightforward microbiological explanation for latent bacterial infections and indeed for the lack of a clinical response to antimicrobial agents shown to be effective against growing cells *in vitro*. It is very likely that many more diseases will have a *microbial* aetiology than we currently recognise (Davey and Kell, 1996).

Again, host cytokines can play a key role in ending latency and beginning the development of an active state of such diseases, especially when the initial concentration of infecting bacteria is likely to be very low. For example, (although the many and often conflicting roles of the various cytokines here remain to be elucidated (Flynn & Bloom, 1996; Henderson *et al*., 1996; Rook & Hernandez-Pando, 1996; Rook & Hernandez-Pando, 1996; Toossi, 1996)), transforming growth factor (TGF β−1) accelerates the growth of *M. tuberculosis* in monocytes and may be important in the pathogenesis of tuberculosis (Hirsch *et al*., 1994). Incubation of pathogenic mycobacteria (*M. tuberculosis* and *M. avium*) in the presence of epidermal growth factor resulted in the acceleration of bacterial growth within macrophages which might have a role in bacterial multiplication in both granulomatous and necrotic tissues (Bermudez, *et al*., 1996). The decrease of insulin concentration in the blood of diabetics results in a significant proliferation of *Ps. pseudomallei* - the causative agent of melioidosis (Woods, *et al*., 1993). Similarly, transferrin is essential for *Francisella tularensis* growth and survival in the acidic vacuole of murine macrophages (Fortier *et al*., 1995). Specific signal(s), derived from the host or the invader, may help to resuscitate such dormant forms to active states and promote their growth, just as was found under laboratory conditions (Wai *et al*., 1996; Whitesides & Oliver, 1997). Indeed, the resuscitation of nonculturable forms of a pathogen,

Legionella pneumophilia, in cells of an amoeba (*Acanthamoboe castellanii*) which serves as a host for this bacterium was recently reported (Steinert *et al*., 1997) (although co-cultivation of 'nonculturable' *L. pneumophilia* with another ciliate, *Tetrhymena pyriformis*, failed to recover viable cells (Yamamoto *et al*., 1996)). Possibly intrinsic *bacterial* cytokines can also be involved in the process of transition to and from dormant (latent) state as well as in cell growth and thus the development of infection.

Given the well-known problems of emerging antibiotic resistance (Bloom & Murray, 1992; Davies, 1994; Duncan, 1998; Murray, 1991; WHO, 1997), the recognition that growth factors can control the development and multiplication bacteria opens the possibility of finding new targets for antibacterial agents, which should not be toxic for animals and may be chosen (or otherwise) to be reasonably selective among species. Indeed, the protein sequence of *M. luteus* Rpf revealed strong similarities only within a fairly narrow family of Gram-positive bacteria, and not with other bacteria and higher organisms of known genome sequence. Equally, appropriate agonists may be of value in decreasing the time of treatment in medically important organisms displaying latency.

Another possibility to exploit bacterial cytokines is to make "attenuated" bacterial strains suitable for vaccination. Knowing the nature of the growth factor(s) for the particular bacteria we might be able to make a strain lacking this by constructing a defined knockout mutant either directly in respect to the growth factor if it is a protein, or indirectly by knocking out a step in the biosynthetic pathway. Such strains could be cultivated easily on suitable media in the presence of exogenous growth factor *in vitro* while being unable to grow *in vivo* because the exogenous growth factor will be absent. Finally, antibodies or vaccines targeted against bacterial virulence (Balaban *et al*., 1998) or growth factors themselves may prove useful therapeutic agents.

9. "AS-YET-UNCULTURED" BACTERIA

While there are well known organisms which have not yet been cultured axenically (e.g. *M. leprae, Tropheryma whippelii* - the causual agent of Whipple's disease (Relman *et al*., 1992) - or the agent of human ehrlichiosis (Fredricks & Relman, 1996)), as well as many organisms present in environmental samples (Amann *et al*., 1995) which are clearly the progeny of viable cells, it is now known that the bacterial species which have been cultivated in laboratory conditions may actually represent only a *very small* fraction (0.01-0.1%) of those occupying our biosphere. These "as yet uncultured" cells are *operationally* nonculturable but in many cases they can be recognised by molecular and cytological methods such as rRNA analysis and *in situ* hybridisation to be previously undetected and unclassified (Amann, *et al*., 1995; Fredricks and Relman, 1996; Head *et al*., 1998). The reasons for the purported "unculturability" of these bacteria are not understood, but numerous attempts at cultivation by using different media, oxygen tensions etc. leads to the conclusion that the formulation of standard nutritional media alone may be not enough to cultivate them in the laboratory. But we would especially stress that in many cases they do not form phylogenetically distinct branches from known, cultured organisms (McVeigh *et al*., 1996).

It is particularly worth mentioning that the media we tend to use have evolved in concert with our recognition of what organisms require in order to grow, such that certain media will inevitably cause slow-growers on such media to be outgrown by fast-growers. One solution to this problem is the exact equivalent of the MPN method, which allows one to culture such organisms by *dilution to extinction*, as particularly well exemplified by the work of Schut and colleagues (Button *et al*., 1993; Schut *et al*., 1993; Schut *et al*., 1997; Schut *et al*., 1997).

In addition, it is of course entirely plausible (and even likely (Kaprelyants and Kell, 1996)) that these "uncultured microorganisms" actually need some growth factors for their cultivation *in vitro*. The example of the cultivation of *M. luteus* on succinate minimal medium is a good simulation of this

situation, when apparently “uncultured “ bacteria (on SMM) became culturable in the presence of a cytokine (Rpf) (Fig.6). Also, washed *M. smegmatis*, which revealed an absolute requirement for an autocrine growth factor to be cultivated in appropriate medium (Table 2) could be considered as a model for a more “natural” population of starved bacteria in soil, which might need a cytokine to start growth (and clearly such proteinaceous factors may be degraded over time in nature).

10. MECHANISMS OF BACTERIAL CELL MULTIPLICATION AND CYTOKINES

While the mechanisms responsible for division of bacterial cells are not fully understood, recent findings on the structure, regulation and functioning of the cell division machinery give some insight into the problem of cytokinesis. Genetic studies have revealed some of the central events of cell division, such as formation of the division septum and initiation of division which are controlled by several gene products. In particular, the gene cluster *ftsQAZ* is most important for cell septation (Vicente & Errington, 1996). FtsZ, an abundant bacterial protein from this gene cluster, plays a crucial role in bacterial division, and is a homologue of the eukaryotic tubulin which is able to undergo GTP-driven polymerization to produce long thin sheets of protofilaments. This polymer is evidently a part of the system (motor) which drives septa to the appropriate place of the cell (Erickson, 1997). The expression of the *FtsQAZ* cluster, in turn, is regulated by SdiA protein which stimulates transcription of *Fts* genes via promoter P_2. Interestingly *SdiA* has strong homology with *luxR* - the key gene in controlling cell-density regulation luminescence of some *Vibrio* spp. via the secretion of low molecular weight inducers belonging to the N-acyl homoserine lactone (HSL) family (Garcia-Lara *et al*., 1996; Sitnikov *et al*., 1996). It is obviously possible that SdiA might function similarly to LuxR and (therefore) cell division in bacteria may also be controlled by cell density via a specific inducer.

Thus, Sitnikov *et al*. (Sitnikov, *et al*., 1996) reported that conditioned medium of growing *E. coli* indeed stimulates trascription from the SdiA-dependent promoter P_2 during cell growth in early log phase. The nature of the inducer(s) in conditioned medium was not clarified; however different known inducers from the HSL family (but not non-acylated HSL) also have a positive effect on the same transcription process. The authors interpreted these results in favour of a "quorum sensing" mechanism for the regulation of *E. coli* growth (Sitnikov, *et al*., 1996). However in similar experiments, Garcia-Lara *et al*. did not find stimulation of the same promoter (SdiA-P_2) in early log phase by conditioned medium (Garcia-Lara, *et al*., 1996). In contrast, they reported a down regulation of this promoter in mid- to late-log phase. The extracellular factor responsible for this regulation was soluble, and resistant to heating, but neither HSL nor OHSL could substitute for the factor from conditioned medium. The evident discrepancy in these results could be based on the use of different media, supporting lower growth rates in the case of the studies reported by Sitnikov *et al*. (Garcia-Lara, *et al*., 1996). At all events, the above results serve to suggest one possible link between the extracellular control of cell multiplication and the molecular mechanisms responsible for the cell division machinery.

11.CONCLUDING REMARKS .

Bacterial cytokines: what for?

Why and under what circumstances should the multiplication of bacterial cells in culture need to be controlled by secreted pheromones or cytokines? While the general rôle of growth factors and cytokines in controlling the development of differentiated eukaryotic systems is clear, the role of bacterial cytokines is less evident, although the advantages of *differentiation* in prokaryotic culture are relatively easy to rationalise (Davey and Kell, 1996; Koch, 1987; Koch, 1993). In addition, at least teleologically, the advantage of the social behaviour of cells during culture growth could follow

from the context of a general strategy of catabolising substrate (Kell, 1987; Westerhoff *et al*., 1983) and of increasing biomass as quickly as possible, and in the rapid response to changing nutritional and other circumstances (Kell, *et al*., 1995). In particular the role of the cytokine may be in "monitoring" the relevant ecological niche for the presence of substrates appropriate for culture development. In the case of "rich" environments, "starter" cells will pass the signal in the form of a cytokine, resulting in an autocatalytic enchancement of multiplication. Hence, bacterial cytokines may play a regulatory role at the level of the population by controlling the balance between multiplication rate and the availability of substrates.

This suggested role of bacterial cytokines may be especially important for nonmotile bacteria (e.g. "branching" bacteria like mycobacteria and streptomycetes) which, in contrast to motile bacteria, are deprived of an efficient machinery (taxis) for searching for locations appropriate for multiplication. Starting with random growth, cells in a "successful" branch (surrounded by enough nutrients) may promote growth in this direction by the secretion of cytokines. Otherwise diffusing growth factors may specifically target to appropriate substrates (e.g. the surface of an infected host cell), providing bacterial cell multiplication in close vicinity to the host and increasing the probability of invasion. Interestingly, the *M. luteus* Rpf C-terminal domain (and a putative Rpf of *M. tuberculosis)* contains regions with significant similarity to p60, a *Listeria monocytogenes* protein required for this microorganism's adherence to and invasion of mouse fibroblasts (Bubert *et al*., 1992). Of course, more experimental evidence should be accumulated to verify these hypotheses.

We also recognise that there may be a selective advantage for an organism to engage in full growth of all cells only if conditions are favourable for a certain period of time. During this 'probing time' a very few cells - 'sentinels' (Postgate, 1995) - might be able to grow but their growth will provide a growth stimulus in the form of Rpf for the rest of the population. If for any reason (the presence of antibiotics or a change to harmful conditions) growth of the sentinels is stopped, the bacterial

cytokines will not be produced to any extent and the cells which had remained in the resting state will stay protected as a result of the stress resistance which typically accompanies the possession of a stationary phase physiology (Hengge-Aronis, 1996).

Biological unity and evolution

It seems obvious that many or most phenomena thought characteristic of higher eukaryotes are likely to have evolved from older clades, including ancestral prokaryotes and/or archaea (Forterre, 1997), such that phenomena observable in one group may be expected to have recognisable counterparts in others (and we may expect this to be confirmed in the post-genomic era). Thus, we now see how the long-standing recognition of the importance of pheromones in effecting communication between higher organisms (Eisner & Meinwald, 1995) is now being extended to prokaryotes (see (Stephens, 1986) and many other contributors to this volume).

Regarding the phenomena of growth, cell cycle progression, stasis and dormancy, we note that even tumours can enter a state of dormancy, which can be reversed by immunodepression (Wheelock *et al.*, 1981). Possibly the immune system normally keeps the growth factors required by the tumour at a sufficiently low level, and there is also evidence that the addition of appropriate antibodies will induce a long-lived dormant tumour state (Racila *et al.*, 1995; Yefenof *et al.*, 1993), although it appears that here the antibody reagents themselves act as signal transduction agonists.

We have therefore sought to stress in this review that, especially in view of our own discovery of bacterial cytokines, and the recent findings that polypeptides with potent activity in promoting cell division may be found in higher plants (Matsubayashi and Sakagami, 1996; Matsubayashi, *et al.*, 1997; van de Sande, *et al.*, 1996), in multicellular invertebrates (Ottaviani, *et al.*, 1996) and even in unicellular eukaryotes such as ciliates (Christensen, *et al.*, 1998; Luporini, *et al.*, 1995), a non-

eukaryotic origin for such activities is most likely. Indeed, the *roles* of polypeptide hormones derived form larger precursors by proteolytic activity exhibit significant similarities between plants and animals (Bergey *et al*., 1996; Schaller & Ryan, 1996), and this type of signal processing may thus have occurred rather early in evolution.

Bacterial culture as a social phenomenon

Thus, it is now clear that cells in bacterial cultures (as a socially organised system) are not independent but are talking to each other using specific chemical messages for many processes (Kell, *et al*., 1995; Lenard, 1992), which clearly include multiplication. From this point of view a bacterial culture resembles a tissue cell culture, and a bacterial colony (Shapiro, 1995), or even the entire microbial world (Mathieu & Sonea, 1995; Mathieu & Sonea, 1996), may be considered as an organism composed of physiologically distinct tissues.

However our knowledge of the control of bacterial cell growth by secreted cytokines is very limited, due to both conceptual/terminological problems (Kell, *et al*., 1998) and more straightforward experimental difficulties. Indeed, for unstressed (uninjured) bacteria and optimal growth media, the "self-promoting" mode of culture growth can be masked due to the high rate of production of growth factors and the high sensitivity of the cells to these pheromones, which can result in the successful multiplication of one isolated bacterial cell to form a culture in a test tube or a colony on agar-solidified medium. As with unicellular eukaryotes (Christensen, *et al*., 1998), only under unfavourable conditions (poor medium, low cell concentration in the inoculum, starved cells, or their combination) is this behaviour visible. These circumstances should be taken into account for future work aimed at generalising the idea of bacterial cytokines to other prokaryotes and nominally undifferentiated, unicellular microorganisms.

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TABLE 1.

Some autocrine/paracrine (pheromone) substances and some animal hormones which have been shown to stimulate the growth of bacteria and other unicellular organisms

Autocrine factors

Organism	***Role***	***Chemical nature***	***Reference***
Bacillus spp.	reduction of lag phase, permit growth from small inoculum	siderophores	(Lankford, *et al*., 1957; Lankford, *et al*., 1966)
Euplotes raikovi (ciliate)	growth promotion, mating factor	secreted polypeptides	(Vallesi, *et al*., 1995)
Micrococcus luteus	resuscitation and stimulation of growth after dormancy	secreted protein, 19kDa	(Mukamolova, *et al*., 1998a)
Nitrosomonas europea	reduction of lag phase	N-acyl homoserine lactone	(Batchelor, *et al*., 1997)
Paramecium tetraurelia (ciliate)	stimulation of growth	secreted protein 17kD	(Tanabe, *et al*., 1990)
Pasteurella (Francisella) tularensis	permits growth from small inoculum	not known, low MW	(Halman, *et al*., 1967; Halman and Mager, 1967)
Xanthomonas maltophila	stimulation of growth	chorionic gonadotropin-like ligand, membrane bound protein 48kD	(Carrell *et al*., 1993; Grover, *et al*., 1995)

(TABLE 1, CONTD.)

Mamalian hormones and cytokines

Escherichia coli	interleukins	(Denis, *et al*., 1991; Denis, *et al*., 1991; Porat, *et al*., 1991)
Escherichia coli	granulocyte-macrophage colony-stimulating factor	(Denis, *et al*., 1991)
Escherichia coli and other Gram-negative bacteria	catecholamines	(Lyte, 1992; Lyte and Ernst, 1992)
Giardia lamblia	human insulin-like growth factor (IGF-II)	(Lujan *et al*., 1994)
Mycobacterium avium	interleukin-6 epidermal growth factor	(Bermudez, *et al*., 1996; Denis, 1992)
Mycobacterium tuberculosis	transforming growth factor-beta-1 epidermal growth factor	(Bermudez, *et al*., 1996; Hirsch, *et al*., 1994)
Neurospora crassa	insulin	(McKenzie, *et al*., 1988)
Saccharomyces cerevisiae	insulin	(Berdicevsky & Mirsky, 1994)
Streptococcus (Enterococcus) faecalis	serotonin	(Strakhovskaya, *et al*., 1993)
Tetrahymena thermophila	lipids, alcohols, porphyrins, insulin, bovin serum albumin	(Christensen, *et al*., 1998; Christensen, *et al*., 1995; Wheatley, *et al*., 1993)
Tetrahymena pyriformis	α2-macroglobulin	(Hosoya *et al*., 1995)

Table 2. Purified *M. luteus* Rpf stimulates growth of mycobacteria

Organism	Bacterial growth†	
	Rpf omitted	Rpf added
M. tuberculosis H37Ra	1.3 ± 1.9 (5)	110 ± 32 (5)
M. tuberculosis H37Rv	1.5 ± 2 (4)	45 ± 28 (4)
M. avium	0 (3)	>300 (3)
M. bovis (BCG)	0 (5)	54 ± 38 (5)
**M. smegmatis*	0 (8)	225 ± 44(8)
Mycobacterium kansasii	2.5 ± 2.5 (3)	90 ± 77 (3)

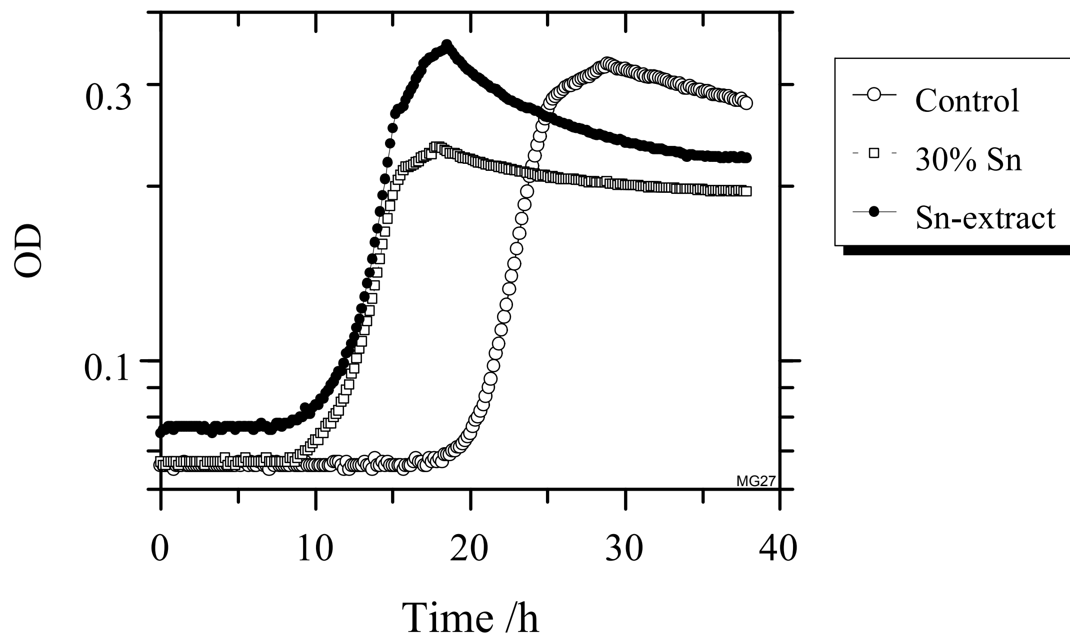
†Growth was estimated microscopically (magnification times 600) after 14 days of incubation; ca. 50 μl of each culture was fixed, stained using Ziehl-Neelsen reagent and counted. Values in the body of the Table are average numbers of cells in a microscope field (10-20 fields counted) ± standard deviation with the number of independent determinations in parentheses. Rpf (after elution from the Mono Q column and dialysis) was used at a concentration of ca. 40 pMol L^{-1}; activity was lost after either trypsin treatment, heating (autoclaving) or filtration through a 12 kDal cutoff membrane. *washed cells of *M. smegmatis* were used for these experiments (Mukamolova, *et al.*, 1998a).

Table 3. Viable count of *M.luteus* estimated by two methods
(for experimental details see text).

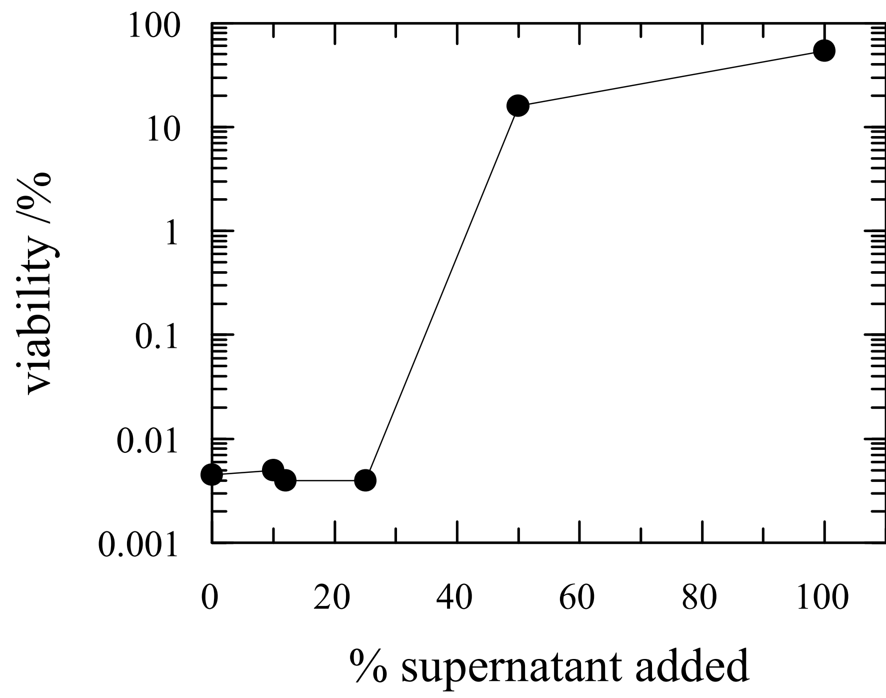
treatment, medium used	**cfu cells ml^{-1}**	**MPN cells ml^{-1}**	**MPN in presence of Rpf, cells ml^{-1}**
washed cells, LMM (5 times)	8.10^8 (BrothE, LMM)	$1.5\ .10^6$ **	5.10^8*
untreated cells, SMM	$3.7\ 10^9$ (BrothE)	$4\text{-}7.10^3$	$5.5\ 10^8$ *
stationary phase cells (100h), LMM	10^8 (BrothE, LMM)	10^5	7.10^8

*recombinant Rpf (3.3 ng ml $^{-1}$)

** poor growth



Kaprelyants *et al*. Fig 1



Kaprelyants *et al*. Fig 2

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g2052146    mlrlvvgalllvlafaggyavaacktvtltvdgtamrvttmksrvidive   50
g2052146    engfsvddrddlypaagvqvhdadtivlrrsrplqisldghdakqvwtta  100
g2052146    stvdealaqlamtdtapaaasrasrvplsgmalpvvsaktvqlndgglvr  150

g2052146    tvhlpapnvagllsaagvpllqsdhvvpaatapivegmqiqvtrnrikkv  200
g2791490    ------------------mpvgwlwrartakgttlknarttliaaaiagt   32

g2440090    ------------------------------------------msesyrkl    8
e1254009    -----------------------------------------msgrhrkpt    9
g2052146    terlplppnarrvedpemnmsrevvedpgvpgtqdvtfavaevngvetgr  250
MlutZ96935  -----------------------------------------mtlfttsat    9
MSGB38COS   --------------------------------------mpgemldvrklc   12
g2225976    ------------mhplpadhgrsrcnrhpisplslignisatsgdmssmt   38
g1655671    -------------------------------mtpglltagagrprdrca   19
g2791490    lvttspagianaddagldpnaaagpdavgfdpnlppapdaapvdtppape   82

g2440090    ttssiivakitftgamldgsialagqaspatdsEWDQVARCESGGNWSIN   58
e1254009    tsnvsvakiaftgavlggggiamaaqataatdgEWDQVARCESGGNWSIN   59
g2052146    lpvanvvvtpaheavvrvgtkpgtevppvidgsIWDAIAGCEAGGNWAIN  300
MlutZ96935  rsrratasivagmtlagaaavgfsapaqaatvdTWDRLAECESNGTWDIN   59
MSGB38COS   klfvksavvsgivtasmalststgmanavprePNWDAVAQCESGRNWRAN   62
g2225976    riakpliksamaaglvtasmslstavahagpsPNWDAVAQCESGGNWAAN   88
g1655671    rivctvfietavvatmfvallglstisskaddIDWDAIAQCESGGNWAAN   69
g2791490    dagfdpnlppplapdflsppaeeappvpvaysVNWDAIAQCESGGNWSIN  132

g2440090    TGNGYLGGLQFSQGTWASHGGGEYAPSAQLATREQQIAVAERVLATQGSG  108
e1254009    TGNGYLGGLQFTQSTWAAHGGGEFAPSAQLASREQQIAVGERVLATQGRG  109
g2052146    TGNGYYGGVQFDQGTWEANGGLRYAPRADLATREEQIAVAEVTRLRQGWG  350
MlutZ96935  TGNGFYGGVQFTLSSWQAVGGEG---YPHQASKAEQIKRAEILQDLQGWG  106
MSGB38COS   TGNGFYGGLQFKPTIWARYGGVG---NPAGASREQQITVANRVLADQGLD  109
g2225976    TGNGKYGGLQFKPATWAAFGGVG---NPAAASREQQIAVANRVLAEQGLD  135
g1655671    TGNGLYGGLQISQATWDSNGGVG---SPAAASPQQQIEVADNIMKTQGPG  116
g2791490    TGNGYYGGLRFTAGTWRANGGSG---SAANASREEQIRVAENVLRSQGIR  179

g2440090    AWPACGHGLSGPSLQEVLPAG--MGAPw----INGAPAPLAPPPPAEPAP  152
e1254009    AWPVCGRGLSNATPREVLPASaaMDAPldaaaVNGEPAPLA-PPPADPAP  158
g2052146    AWPVCAaragar--------------------------------------  362
MlutZ96935  AWPLCSQKLgltqadadagdvdateaapvavertatvqrqsaadeaaaeq  156
MSGB38COS   AWPKCGAASDLPITLWSHPAQGVKQIINDIIqmgdttlaaialngl----  155
g2225976    AWPTCGAASGLPIALWSKPAQGIKQIINEIIwagiqasipr---------  176
g1655671    AWPKCSscsqgdaplgslthiltflaaetggcsgsrdd------------  154
g2791490    AWPVCGrrg-----------------------------------------  188

g2440090    pqppadnf-----------------------PPTPGDVPSPLarp-----  174
e1254009    pvelaandlpaplgeplpaapadpappadlaPPAPADVAPPVelavndlp  208
MlutZ96935  aaaaeqavvaeaetivvksgdslwtlaneyeveggwtalyeankgavsda  206

e1254009    aplgeplpaapadpappadlappapadlappapadlappapadlappvel  258
MlutZ96935  aviyvgqelvlpqa------------------------------------  220

e1254009    avndlpaplgeplpaapaelappadlapasadlappapadlappapaela  308
e1254009    ppapadlappaavneqtapgdqpatapggpvglatdlelpepdpqpadap  358
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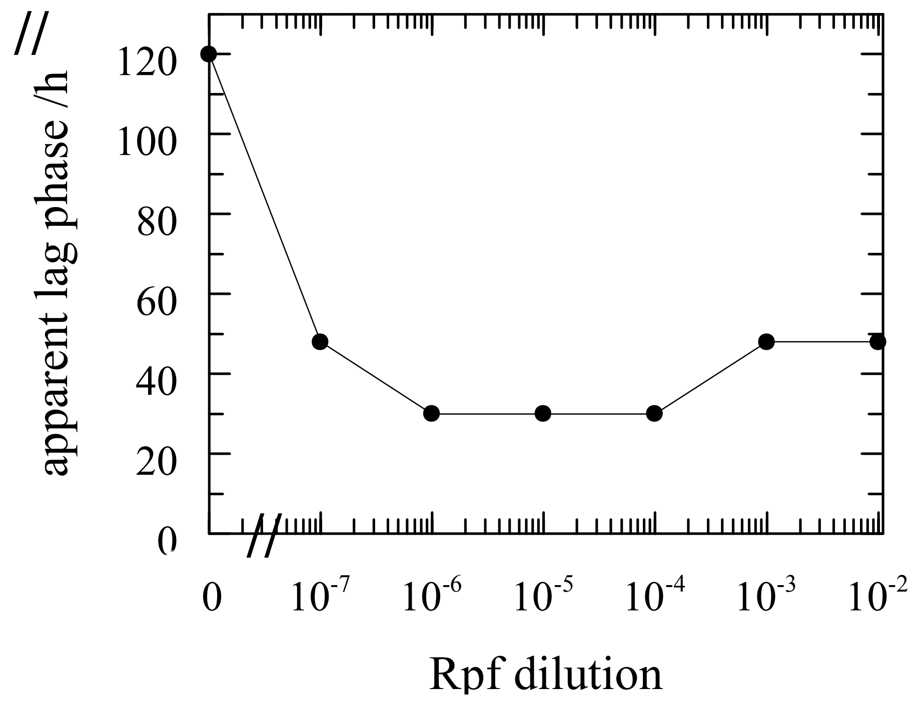
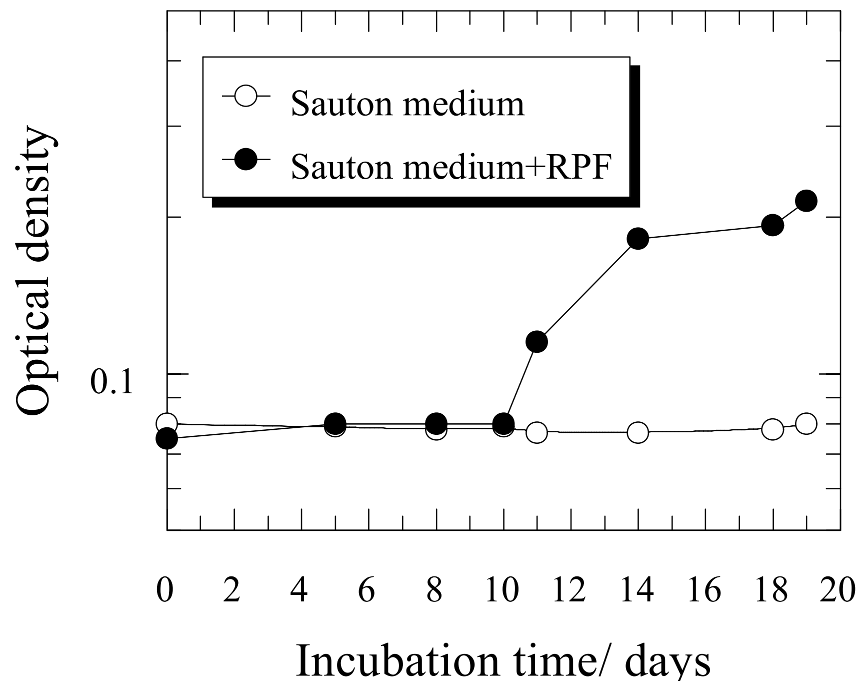
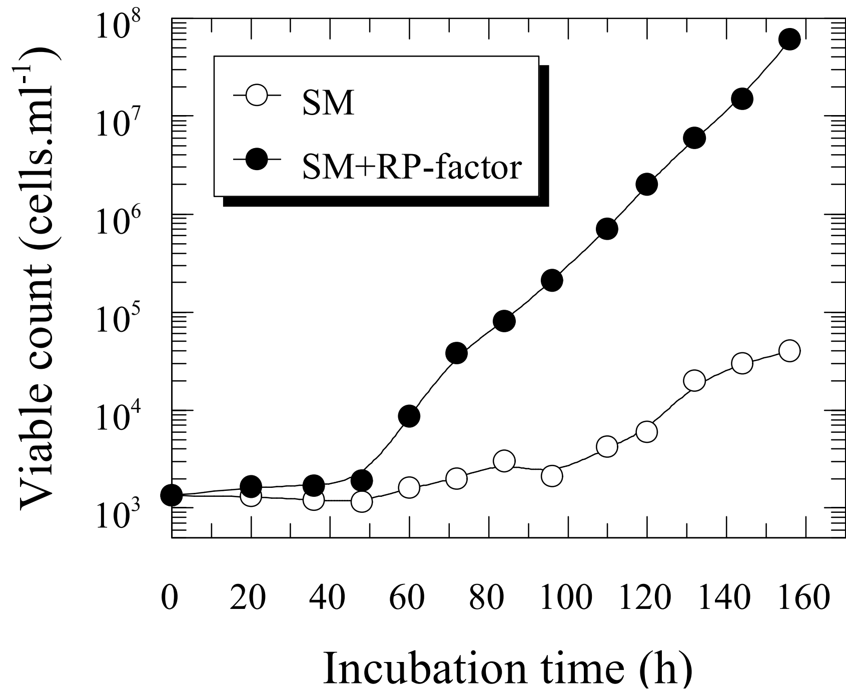
Kaprelyants *et al*. Fig 3



Kaprelyants *et al*. Fig 4



Kaprelyants *et al*. Fig 5



Kaprelyants *et al*. Fig 6

LEGENDS TO FIGURES

Fig 1. Growth of a diluted 50-day-old stationary-phase culture of *Escherichia coli* ZK126 (W3110) in a MOPS-buffered minimal medium with the addition of 30% (v/v) supernatant (Sn) of a 2-day stationary culture of the same organism, or an addition of a crude extract of the same supernatant (Sn-extract), added in a corresponding concentration; control: untreated suspension. Growth was measured in a Bioscreen Microbiological Growth Analyser as in (Mukamolova, *et al*., 1998a). DHW and DBK, unpublished observations.

Fig 2. Effect of culture supernatant on the resuscitation of dormant cells of *Micrococcus luteus*. *M. luteus* was starved for 3.5 months and its viability assessed using an MPN assay as described (Kaprelyants, *et al*., 1994). Supernatants were taken from a batch culture of the organism grown to an OD of 2, slightly before the beginning of stationary phase and mixed in the stated proportion with a lactate minimal medium containing 0.05 (w/v) yeast extract (Kaprelyants, *et al*., 1994).

Fig 3. ***M. tuberculosis* and *M. leprae* contain genes whose products are similar to Rpf.** Multiple sequence alignment of *M. luteus* Rpf (Z96935) with predicted gene products from *M. tuberculosis* g2052146 (MTCI237.26), g2791490 (MTV008.06c), g1655671 (MTCY253.32), g2225976 (MTCY180.34), e1254009 (MTV043.60c), and *M. leprae* g2440090 (MLCB57.05c), MSGB38COS (L01095, nt 12292-12759). Conserved blocks are in upper case, residues conserved or conservatively substituted in five or more sequences are in bold and predicted signal sequences are underlined.

Fig 4. Reduction of the apparent lag phase of viable cells of *M. luteus* by purifed recombinant Rpf. For experimental details see (Mukamolova, *et al*., 1998a). A dilution factor of 10^0 corresponds to 250 µg Rpf ml^{-1}.

Fig 5. Effect of *M. luteus* RPF on the growth of *Mycobacterium bovis* in batch culture. *M. bovis* was grown in Sauton medium to which a 500-fold dilution of RPF was either added (closed circles) or not (open circles). The inoculum was ca 1.10^5 $cells.ml^{-1}$, and the OD shown is the average of 10 tubes.

Fig 6. Effect of *M. luteus* Rpf on the growth of *M.luteus* in batch culture in succinate minimal medium. *M. luteus* was grown in broth E until stationary phase and inoculated into succinate medium (SM) (Mukamolova, *et al*., 1998a). Flasks (20 ml) with SM were inoculated with approximately 1000 $cells.ml^{-1}$ and growth was monitored by sampling aliquots and plating them out on agar plates supplemented with Lab M nutrient broth and incubated at 30°C. In some cases (closed circles) Rpf was added to a final concentration of 4 ng ml^{-1} .

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