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Stimulation of the multiplication of *Micrococcus luteus* by an autocrine growth factor

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Abstract Viable cells of *Micrococcus luteus* secrete a proteineous growth factor (Rpf) which promotes the resuscitation of dormant, nongrowing cells to yield normal, colony-forming bacteria. When washed *M. luteus* cells were used as an inoculum, there was a pronounced influence of Rpf on the true lag phase and cell growth on lactate minimal medium. In the absence of Rpf, there was no increase in colony-forming units for up to 10 days. When the inoculum contained less than 10^5 cells ml^{-1}, macroscopically observable *M. luteus* growth was not obtained in succinate minimal medium unless Rpf was added. Incubation of *M. luteus* in the stationary phase for 100 h resulted in a failure of the cells to grow in lactate minimal medium from inocula of small size although the viability of these cells was close to 100% as estimated using agar plates made from lactate minimal medium or rich medium. The underestimation of viable cells by the most-probable-number (MPN) method in comparsion with colony-forming units was equivalent to the requirement that at least 10^5 cells grown on succinate medium, 10^3 cells from old stationary phase, or approximately 10–500 washed cells are required per millilitre of inoculum for growth to lead to visible turbidity. The addition of Rpf in the MPN dilutions led to an increase of the viable cell numbers estimated to approximately the same levels as those determined by colony-forming units. Thus, a basic principle of microbiology – "one cell-one culture" – may not be applicable in some circumstances in which the metabolic activity of "starter" cells is not sufficient to produce enough autocrine growth factor to support cell multiplication.

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Introduction

Tissue cultures of cells taken from higher, differentiated organisms normally need complex growth factors for successful cell division. These factors are nowadays usually referred to as cytokines. Their role is generally understood to involve binding at the cell membrane and the production of second messengers such as cGMP. These serve to activate various signaling pathways and segments of primary metabolism, which may of course include those responsible for their own synthesis (Alberts et al. 1989). By contrast, it is usually assumed in prokaryotic microbiology that each bacterial cell in an axenic culture can multiply independently of other bacteria, provided that appropriate concentrations of substrates, vitamins and trace elements are present in the culture medium. Current laboratory experience seems to be consistent with this in that the development of bacterial colonies from single cells on agar plates is 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 becoming clear that axenic bacterial cultures do not remotely represent a statistically homogeneous population (Koch 1987; Kell et al. 1991; Davey and Kell 1996), these observations are most easily interpreted as being in favour of "autonomous" growth.

However, an increasing body of evidence has highlighted the widespread importance of chemically mediated intercellular communication between bacteria in culture for specific events such as sporulation, conjugation, virulence and bioluminescence. Thus, it is now clear that a variety of different chemical compounds (pheromones) (Stephens 1986) produced as secondary metabolites are responsible for such social behaviour of prokaryotes as

exhibited under conditions of obvious cellular differentiation [for review, see Kaiser and Losick (1993), Swift et al. (1994, 1996), Kell et al. (1995), Greenberg et al. (1996) Dunny and Leonard (1997), Kleerebezem et al. (1997)]. The question then arises as to whether similar types of signaling may be of general significance for cell multiplication in growing bacterial cultures (Kaprelyants and Kell 1996).

Recently we have found that viable cells of *Micrococcus luteus* secrete a factor that promotes the resuscitation of dormant, nongrowing cells to yield normal, colony-forming bacteria (Kaprelyants and Kell 1993; Kaprelyants et al. 1994; Votyakova et al. 1994). The resuscitation-promoting factor (Rpf) is a protein that has been purified to homogeneity (Mukamolova et al. 1998). Analysis of the nucleotide sequence of the *M. luteus* gene encoding Rpf suggests that the form initially secreted is an approximately 19-kDa protein. Five similar genes that can encode apparently secreted proteins have been uncovered in *Mycobacterium tuberculosis* by genome-sequencing projects (Cole et al. 1998), and two are currently known in *Mycobacterium leprae*. Rpf has been expressed in, and purified to homogeneity from, *Escherichia coli.* Picomolar concentrations of recombinant Rpf increase the viable cell count of dormant *M. luteus* cultures at least 100-fold and can also stimulate the growth of viable cells. Rpf also stimulates the growth of several other high G+C, gram-positive organisms including *Mycobacterium avium*, *Mycobacterium bovis* (BCG), *Mycobacterium kansasii*, *Mycobacterium smegmatis* and *M. tuberculosis*. Thus, Rpf is in fact the first example of a bacterial growth factor or cytokine (Mukamolova et al. 1998).

In the present study we describe the conditions under which the dependence of cell multiplication on the secretion of bacterial growth factor(s) may be observed in some bacteria.

Materials and methods

Organisms and media

M. luteus NCIMB 13267 (previously described as "Fleming strain 2665") was grown aerobically at 30 °C in shake flasks in lactate minimal medium (LMM) containing 0.5% L-lactate as described previously (Kaprelyants and Kell 1992, 1993). For some experiments, lactate in LMM was replaced by 1% succinate (succinate medium; SMM).

M. smegmatis ("fast" strain; All-Russia State Institute for Control of Veterinary Preparations, Moscow, Russia) was grown in nutrient broth E or in Sauton medium (Connell 1994).

M. tuberculosis (avirulent, "Academia" strain) was obtained from the Physiopulmonology Center (Moscow, Rusia). Bacteria were maintained on Lowenstein-Jensen agar slopes at 37 °C and were grown at 37 °C in liquid Sauton medium supplemented with albumin, glucose, NaCl, 0.6% (w/v) glycerol and 0.05% Tween 80.

M. luteus spent medium preparation

Supernatant was obtained after the centrifugation of late-exponential-phase *M. luteus* cultures (200–1000 ml) grown in LMM. The inoculum consisted of 2% of cells grown in rich medium (Broth E, Amersham) and then washed in LMM lacking lactate. The supernatants were passed through a 0.22-μm filter (Gelman) before use.

Preparation of *M. luteus* Rpf

Rpf was prepared from supernatant by ion-exchange chromatography as described in Mukamolova et al. (1998) to a final concentration of 1–60 μg ml^{-1}.

Determination of *M. luteus* cell viability

Cell viability was determined by plating or by a most-probable-number (MPN) assay. For plating, plates consisting of 1.3% Nutrient Broth E (LabM), or LMM or SMM were used. Dilutions were made in quadruplicate with LMM lacking lactate. Plates were incubated at 30 °C for 3–5 days.

The MPN assay was performed using serial dilutions in 2-ml test tubes or in a Bioscreen C optical growth analyzer (Labsystems, Finland) with 100-well plates, each well containing 0.2 ml medium. Various media were used for the MPN assay: LMM, LMM supplemented with 0.05% yeast extract, SMM, and Sauton medium. Cell suspensions were diluted as described (Kaprelyants et al. 1996) in appropriate medium with or without supernatant or Rpf. Growth (optical density) was monitored using a 600-nm filter. Plates or test tubes were incubated at 30 °C with intensive continuous shaking. The calculation of the MPN was based on published tables (Meynell and Meynell 1965)

Chemicals

Nutrient Broth E, yeast extract and agar were obtained from Lab M (Difco), whilst L-lactate (Li salt) and succinate were obtained from Sigma. Other chemicals were of analytical grade and were obtained from Sigma or Fisher.

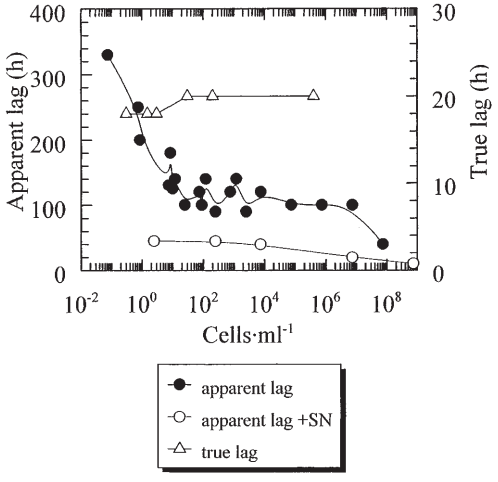

Fig. 1 Effect of inoculum size and the addition of culture supernatant on the regrowth of *Micrococcus luteus*. Cells were grown to stationary phase in lactate minimal medium and reinoculated into lactate minimal medium at the concentrations shown. The length of the lag phase was determined optically (● "apparent" lag) or from CFU count (△ "true" lag). In some cases (○), 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 1:1 with the lactate minimal medium (Kaprelyants et al. 1994)

Table 1 Apparent and true lag phase of *Micrococcus luteus* growth. The resuscitation-promoting factor (Rpf) concentration was 1–10 ng ml^{-1} (*LMM* lactate minimal medium, *SMM* succinate minimal medium)

[a] Limited number of divisions only

Treatment, medium used	True lag phase (h)		Apparent lag phase (h)		Inoculum (cells ml^{-1})
		+Rpf		+Rpf	
Untreated cells, LMM	18	18	110–150	48	200
Untreated cells, SMM	100[a]	48	> 320	150	1000
Washed cells, LMM	> 240	48	> 240	140	250

Results

It is well-known that the duration of the lag phase in batch cultures often depends more-or-less inversely on the size of the inoculum, which, in turn, could reflect the accumulation of some growth inducer(s) secreted by cells during the lag phase. To check this possibility, the "true" inoculum-dependent lag phase (when bacterial growth is monitored by counting viable cells) and the "apparent" lag (when the lag phase is estimated directly from uncorrected optical density traces) need to be studied separately. The latter must, of course, necessarily be "inoculum-size-dependent". Figure 1 shows that the apparent lag phase for an *M. luteus* culture grown on LMM is indeed dependent on the inoculum size, especially in the range of less than one cell ml^{-1}. The true lag phase was almost constant (18–20 h) over a broad range of inoculum size. Addition of supernatant or of isolated Rpf resulted in a significant decrease of the apparent lag phase (Fig. 1), whilst it had almost no effect on the true lag phase . Evidently, supernatant increases the growth rate of bacteria significantly. A similar effect was observed for cells grown in rich medium (Broth E) or in LMM supplemented by yeast extract (0.05%). However the difference between the apparent lag (± Rpf) was visible only when a small inoculum had been used (not shown).

However, when washed cells were used as an inoculum, there was a pronounced influence of Rpf on cell growth. In the absence of Rpf, no increase in CFU up to 10 days was observed (Table 1). Thus, the absence of any Rpf effect on the true lag phase in Fig. 1 may be due to the carryover of a small amount of the putative cytokine by exponentially growing cells, either on their cell surface or in spent medium (notwithstanding the significant dilution). It is worth mentioning that cell washing did not influence the number of CFU on either rich plates or plates with LMM. Similar to that of *M. luteus*, growth of *M. smegmatis* cells also revealed an Rpf-dependence when washed cells had been grown in Sauton medium (Fig. 2).

As an extreme, we found that Rpf supports bacterial growth even in a medium in which *M. luteus* had to date never been cultivated. We used succinate instead of lactate in minimal medium and obtained the results shown in Table 1 and Fig. 3. 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 carryover of some Rpf with the unwashed inoculum. However, the addition of purified Rpf (4 ng ml^{-1}) resulted in cell growth sufficient to form a turbid suspension (Fig. 3). It should be stressed that washed cells did not lose the ability to form colonies on LMM agar, and normal cells can also grow on agar prepared with SMM without added Rpf. It is interesting that the number of CFU per unit volume of culture medium as assessed on plates with succinate medium depended on the total number of cells that were plated out on the agar, changing from 5% to 70% (of the CFU that could be observed on rich agar plates) (Fig. 4).

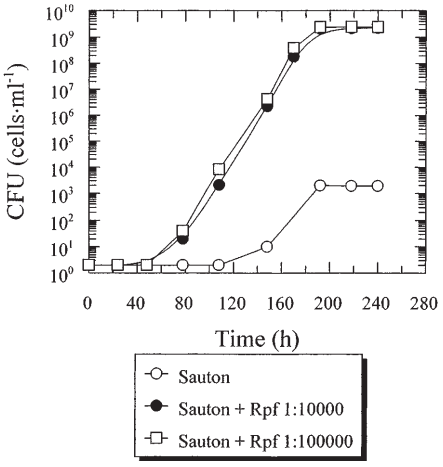

Fig. 2 Effect of *Micrococcus luteus* resuscitation-promoting factor (Rpf) on the growth of *Mycobacterium smegmatis* in batch culture. *M. smegmatis* cells washed five times in Sauton medium before inoculation were grown in 25-ml flasks on Sauton medium at 37 °C with agitation. In some cases, Rpf was added in dilutions of 1 : 10,000 or 1 : 100,000 (initial concentration of Rpf was 60 µg ml^{-1}) Growth was monitored by sampling aliquots and plating them out on agar plates supplemented with Broth E and incubated at 37 °C

Earlier we noticed that *M. luteus* cells taken from a long stationary phase as an inoculum grew very poorly on LMM. We checked the culturability of such cells by two methods: by plating out on agar plates, and by an MPN assay. Figure 5 shows that the incubation of cells for 100 h in the medium in which they had grown to stationary phase resulted in significant differences between the MPN count and CFU (the latter was almost identical for both LMM and rich-medium plates). Underestimation of viable cells by the MPN method means that at least 1000 viable cells (as judged by CFU) should be present in a test tube if they are to go on to produce visible growth. When Rpf was added to the MPN dilutions, the estimation of the

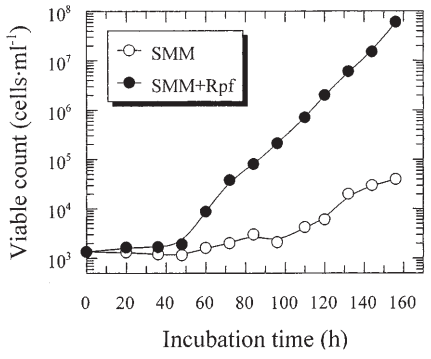

Fig. 3 Effect of *Micrococcus luteus* resuscitation-promoting factor (Rpf) on the growth of *M. luteus* in batch culture in succinate minimal medium (SMM). *M. luteus* was grown in Broth E until the end of the exponential growth phase, washed once and resuspended in SMM. Flasks (20 ml) with SMM were inoculated with approximately 1,000 cells per milliliter, and growth was monitored by sampling aliquots and plating them out on agar plates supplemented by Broth E and incubated at 30 °C. In some cases (●), Rpf was added at a dilution of 1:1,000 (initial concentration, 1 μg ml^{-1})

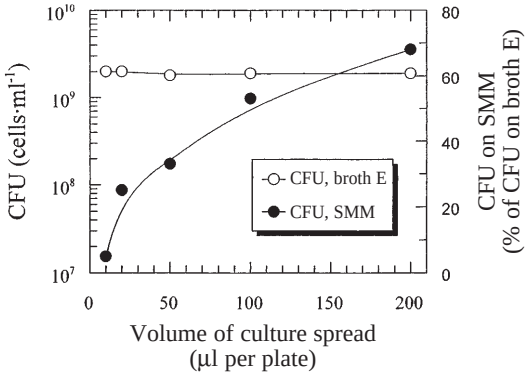

Fig. 4 Dependence of CFU number on solid medium supplemented by rich (Broth E) medium or by succinate minimal medium (SMM) on the initial concentration of cells per plate. Before plating, cells were grown in Broth E until the end of the exponential phase. Cells were diluted 10^6-fold before plating. Plates were incubated for up to 20 days at 30 °C

number of viable cells was similar to that determined by plate counts, which demonstrates the ability of one "old" cell to grow in a test tube in the presence of Rpf.

The results of our study of the culturability of *M. luteus* cells obtained by the two methods are summarised in Table 2. The underestimation of viable cells by the MPN method (in comparison with viable counts judged on plates) after cell washing or using old cells or cells grown in SMM indicates that more than one cell must initially be present per well if visible growth is to be produced. Again, when Rpf was added to the MPN dilutions, the estimation of the number of viable cells was similar to that determined by plate counts (Table 2). Such a comparison demonstrates that at least 10^5 cells grown on succinate medium, 10^3 of cells from old stationary phase, and approximately 10–500 washed cells are required per millilitre of inoculum for visible growth. At the same time, performance of the MPN assay for "washed" and "old" cells in

Table 2 Viable count of *Micrococcus luteus* estimated by two methods. *M. luteus* cells were grown until stationary phase in lactate minimal medium (LMM), and their viability was estimated by MPN in well plates [using succinate minimal medium (SMM) as dilution medium] and by CFU ("untreated" cells) The same cells were either washed several times with LMM and resuspended in LMM ("washed cells") before MPN estimation (using LMM as dilution medium) and CFU estimation or they were held in stationary phase for more than 100 h before estimation of their viability [as in the case of ("stationary"). In some cases, resuscitation-promoting factor (Rpf; 1–10 ng ml^{-1}) was added to each well or tube for the MPN assay

Treatment, medium used for the MPN assay	CFU (cells/ml)	MPN (cells/ml)	MPN in presence of Rpf (cells/ml)
Washed (5 times) cells, LMM	$8 \cdot 10^8$ (Broth E, LMM)	$1.5 \cdot 10^6$ [a]	$5 \cdot 10^8$
Washed (3 times) cells, LMM	$1.3 \cdot 10^9$	$1.5 \cdot 10^8$ [a]	$2.3 \cdot 10^9$
Untreated cells, SMM	$3.7 \cdot 10^9$ (Broth E)	$4.7 \cdot 10^3$	$5.5 \cdot 10^8$
Stationary cells (100 h), LMM	10^8 (Broth E, LMM)	10^5	$7 \cdot 10^8$

[a] Poor growth

rich liquid medium (Broth E) resulted in a viable count similar to the CFU number.

Discussion

The results of the present experiments show that Rpf has a significant influence on the growth of producer cells or cells that secrete Rpf homologues (and, in addition, Rpf increases the final yield of bacterial biomass, as shown in Table 3). However, the observability and character of its action depends (as with the results from any viability estimation; Barer et al. 1998; Kell et al. 1998) on the conditions used in any particular experiment. For example, if untreated cells from the late exponential phase were used to test the activity of Rpf on the lag phase, we found that Rpf mainly influenced the growth rate rather than the duration of the true lag phase, changing only the apparent lag phase (Fig. 1). At the same time, Rpf decreased the true lag phase if washed cells or cells grown in a very inappropriate medium (succinate medium) were used (Table 1). Indeed, it has been known that 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 has been detected only in a poor medium, but not in a rich one (Shida et al. 1977). Similarly, the study of an inoculum-dependent lag for various *Bacillus* spp. has been performed by using poor medium (Lankford et al. 1966). Dagley et al. (1950) have 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. We may suggest that a small amount of Rpf that is carried over by unwashed *M. luteus* cells used as an inoculum can be sufficient to stimulate the initial multi-

Table 3 Effect of resuscitation-promoting factor (Rpf) on the maximum optical density of broth cultures of various high G+C, gram-positive bacteria. For conditions for growing the bacteria, see Material and methods. Rpf concentration was 1–10 ng ml^{-1}(LMM lactate minimal medium)

	No addition	+Rpf
Micrococcus luteus (LMM)	4.8	9.4
Micrococcus luteus (Broth E)	7.5	11.5
Mycobacterium smegmatis (Broth E), washed	2.9–3.1	6.2–7.0
Mycobacterium smegmatis (Sauton), washed	0–0.6	2.4
Mycobacterium tuberculosis (Sauton +Tween)[a]	1.2	2–2.6
Mycobacterium tuberculosis (Sauton+Tween+oleate)[b]	2.5	5.0

[a] Cultivation for 35 days
[b] Cultivation for 30 days

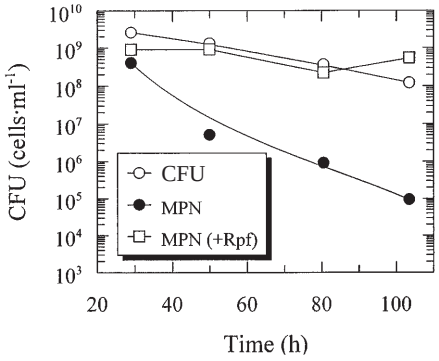

Fig. 5 Estimation of the viable count of *Micrococcus luteus* cells held in stationary phase. When *M. luteus* culture grown in lactate minimal medium (LMM) had reached stationary phase (30 h), agitation was continued at 30 °C for up to 4 days. Periodically the viability of cells by MPN (in LMM) and by CFU (using Broth E or LMM-supplemented agar) was estimated. In some cases, Rpf (concentration, 2 ng ml^{-1} was added to each tube for the MPN assay

plication of cells, while added Rpf further stimulates exponential growth. In the extreme situation, when growth of cells is either absent (washed cells) or occurs only until a limited number of divisions have been made (SMM), Rpf allows cells to grow with a division time of approximately 10 h (for SMM) and 5 h (for LMM). Normal *M. luteus* cells grow on LMM with a division time of 4 h.

We have to stress that washed cells have not lost the ability to form colonies on LMM agar medium, and cells can grow on agar prepared with SMM without 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 by juxtacrine signaling by cell-surface-associated proteins. The dependence of the CFU number on the number of cells spread on SMM agar is also in favour of the importance of cell-cell interactions for their growth (Fig. 4).

The application of two independent methods for the estimation of bacterial viability gives one the possibility of estimating quantitatively how many cells in a liquid medium can support growth in the absence of any externally added growth factor. For various treatments and media the minimal number of *M. luteus* cells required per millilitre for visible growth was from 10^2 to 10^5 (Fig. 5, Table 2), despite the fact that almost all of the cells in these populations were viable and able to form colonies on agar plates. To our knowledge, this is the first detailed demonstration [but see also Votyakova et al. (1994) for resuscitation] of the existence of a "threshold" initial number of bacterial cells that must be present for their further growth, a phenomenon known for nucleated cells (Wheatley et al. 1993; Christensen et al. 1995, 1998). Hence, we may suggest that the basic principle of microbiology – "one cell-one culture" – may not work in some circumstances. The involvement of bacterial growth factors in this phenomenon is likely since the addition of Rpf resulted in very similar viable counts as judged by the two methods. At the same time, the experiments of this study clearly show the benefit of studying "unfavourable" conditions in order to make the dependence of bacterial growth on secreted growth factors or cytokines most visible (Wheatley et al. 1993; Christensen et al. 1998).

The mechanisms responsible for "nonculturability" (in the operational sense; Barer et al. 1998; Kell et al. 1998) of bacterial cells depleted of exogenous Rpf in liquid medium in the above experiments 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, which, in turn, depend on the medium composition, the source of nutrients, etc. A metabolically active cell may have a finite "lifetime" during which it can survive without division. If held in lag phase for a period exceeding this lifetime (e.g. washed *M. luteus* cells in LMM or cells grown in SMM), cell death ensues by mechanisms that remain to be elucidated.

The "self-promoting" mode of bacterial cell growth may be especially important for future attempts to cultivate so-called "uncultured" microorganisms found in nature (Kell et al. 1998) since current formulations of nutritional media may be not enough to bring them into cultivation in the laboratory (Kaprelyants et al. 1999). Another significant implication of the existence of this self-promoting mode of bacterial growth is in infections, when the initial concentration of infecting bacteria is likely to be very low (Smith 1998) and bacterial cytokines may play an important role in the development of an infection. The results obtained in the present study also show that *M. luteus* and *M. smegmatis* cultures behave similarly under conditions that are designed to make growth dependent on externally added Rpf (Fig. 2). As with *M. luteus*, we found a similar "threshold" or "cut-off" phenomenon for old cultures of *M. smegmatis* (G. V. Mukamolova, D. B. Kell, A. S. Kaprelyants, unpublished work). These findings indicate the possible involvement of bacterial cyto-

kines in mechanisms of the latency of diseases caused by pathogenic mycobacteria (Kell et al. 1998; Kaprelyants et al. 1999).

Finally, it is reasonable that the control of bacterial growth by autocrine growth factor(s) may not be limited to *M. luteus* and closely related gram-positive bacteria, but may have general significance (Kaprelyants et al. 1999). However, if the same type of very high sensitivity of the bacterial cells to growth factor is as prevalent as in the present case, appropriate conditions for the visualisation of their activity will need to be established.

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