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Do bacteria need to communicate with each other for growth?

Arseny S. Kaprelyants and Douglas B. Kell

Tissue cultures of cells taken from higher, differentiated multicellular organisms normally need complex growth factors (cytokines) for successful cell division. The role of these factors is thought to involve binding at the cell membrane and the production of second messengers, such as cyclic GMP, which activate various segments of primary metabolism, possibly including those responsible for their own synthesis[1]. By contrast, it is usually assumed that each bacterial cell in an axenic (pure) culture can multiply independently of other bacteria, provided that appropriate concentrations of substrates, vitamins and trace elements are present in the culture medium, and that physicochemical environmental parameters, such as pH, temperature, water activity and oxygen status, are at levels compatible with the growth of the organism.

Current laboratory experience seems to be consistent with this idea: 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 one viable cell will eventually show visible growth or turbidity. Although axenic bacterial cultures are not a homogeneous population[2,3], these observations are most easily interpreted as being consistent with 'autonomous' growth. However, especially in the most probable number method, growth is almost always analysed in the presence of culture supernatants or cell-adherent substances introduced with the inoculum,

It is usually assumed that most prokaryotes, when given appropriate nutrients, can grow and divide in the absence of other cells of the same species. However, recent studies have suggested that, for growth, prokaryotes need to communicate with each other using signalling molecules, and a variety of 'eukaryotic' hormones have been shown to stimulate bacterial growth. These observations have important implications for our understanding of bacterial pathogenicity.

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which may contain potent autocrine growth factors produced by the cells during their previous growth phase that are necessary for the initiation of regrowth or division.

There is increasing evidence for the widespread importance of chemically mediated intercellular communication in bacterial cultures for some events, including sporulation, conjugation, virulence and bioluminescence. A variety of different autocrine chemicals (pheromones[4]) produced as secondary metabolites are responsible for the social behaviour of prokaryotes, under these perhaps specialized conditions involving obvious cellular differentiation (for reviews, see Refs 5–10).

Pheromones differ from nutrients in that they are produced by the organisms themselves, they are active at very low concentrations and, in the absence of prohormones, their metabolism is not necessary for growth (although they may of course ultimately be degraded). Are similar types of signalling of wider significance for cell multiplication in growing bacterial cultures? Here, we outline many examples in which an apparently optimized nutrient medium is insufficient for cell growth, and where there is evidence for the involvement of hormones and pheromones in prokaryotic growth and division.

Starvation, lag phase and outgrowth

Starvation

Much molecular information has recently become available about the physiological changes accompanying
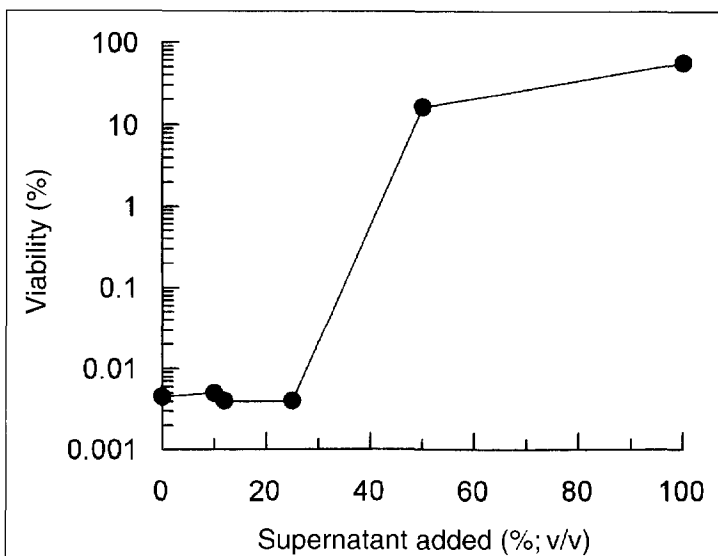
Fig. 1. Effect of culture supernatant on the resuscitation of dormant cells of *Microccus luteus*. *Micrococcus luteus* was starved and its viability assessed using a most probable number assay, as described in Ref. 19. Supernatants were taken from a batch culture of the organism grown to an optical density of 2, slightly before the beginning of stationary phase, and mixed in the proportions shown with a lactate minimal medium containing 0.05 (w/v) yeast extract[19].

bacterial starvation[11,12]. However, much less is known about the processes that occur during the outgrowth of starved cells, and current evidence suggests that the culture is divided into cells that emerge smoothly from the lag phase and cells that do not. Starved marine bacteria[13], soil bacteria[14], *Pseudomonas fluorescens*[15] and *Micrococcus luteus*[16] stop growing after several divisions
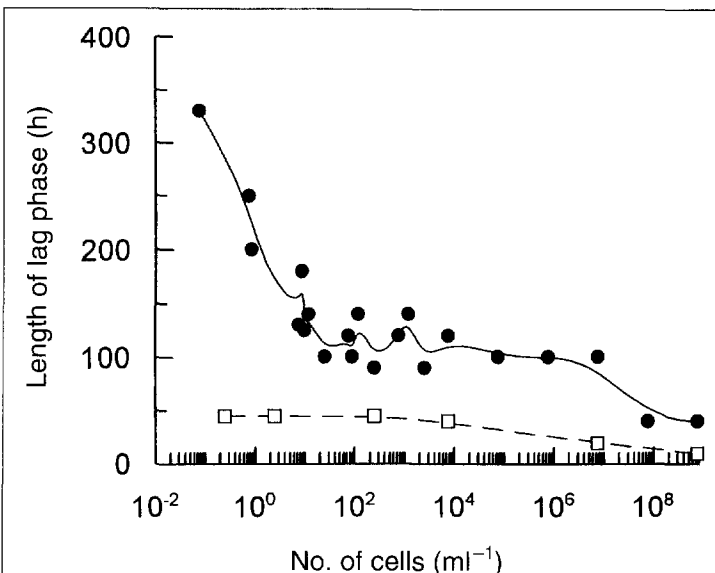
Fig. 2. Effect of inoculum size and the addition of culture supernatant on the regrowth of *Micrococcus luteus*. Cells were grown to stationary phase in lactate minimal medium and reinoculated into lactate minimal medium at the concentrations shown. The length of the lag phase was determined optically (squares, filled circles). In some cases (squares), supernatants were taken from a batch culture of the organism grown to an optical density of 2, slightly before the beginning of stationary phase, and mixed 1:1 with the lactate minimal medium[19].

when cells are incubated in fresh medium. In the case of *M. luteus*, we found that, while cell growth stops, the cells appear to remain metabolically active (maintaining membrane energization), as judged by flow cytometry[17]. One mechanism that may be responsible for causing this cessation of cell growth is an accumulation of inhibitory substances during the growth of previously starved cells[13,18]. For example, we have demonstrated the secretion of a 'killer factor' that inhibits the growth of viable bacteria during the resuscitation of starved *M. luteus* cultures[16].

In these (and probably in other similar) cases, at the beginning of cell growth, starved cells have a reduced ability to excrete the appropriate growth factor(s) needed to stimulate cell division. As a result, only some of the cells in a population can multiply, while the gradual accumulation of toxins formed by nondividing cells eventually prevents cell multiplication. The addition of supernatants from growing *M. luteus* cultures to the starved culture relieves the inability of the cells to divide more than a few times, and allows the resuscitation of cells to normal colony-forming cells[19] (Fig. 1). In other words, starvation may make the cells sensitive to specific chemical inducers of cell division, which are not important under normal growth conditions. More generally, there may be a limited quota of hormone, the initial concentration of which is low in poor media and which degrades over time; this hormone is shared out between cells over generations until its concentration per cell is inadequate for division.

Duration of the lag phase

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[20] (and see later discussion). (In some studies, this 'true' inoculum-dependent lag phase is confused with the 'apparent' lag that occurs when the lag phase is estimated directly from uncorrected optical density traces, which clearly depends on the size of the inoculum.) This might reflect the accumulation of growth inducer(s) secreted by cells during the lag phase, and the addition of supernatant from log-phase bacterial cultures does significantly shorten the inoculum-dependent lag phase in several cases[21-23]. However, an inoculum-dependent lag phenomenon may occur only under a restricted range of conditions: for *Achromobacter delmarvae*, an inoculum-dependent lag occurs in a poor medium but not in a rich one[24]. Similarly, inoculum-dependent lag in various *Bacillus* species has been studied in poor medium[22]. Dagley *et al.* found that the effect of supernatant on the inoculum-dependent lag itself depends on the size of the inoculum: the largest effect from the addition of supernatant was observed with the smallest inoculum[21]. Our own similar data are shown in Fig. 2.

Of course any substance produced by cells in the culture supernatant may have growth-affecting properties; for example, those of CO_2 are well known[25]. Sudden changes in the content of dioxygen and its reactive metabolites (such as peroxide and superoxide) are a particularly potent stress that can be encountered

as organisms are inoculated into or on to different media and, if cells can degrade such inhibitory substances, this will also contribute to the effect of inoculum size on the extent of the lag phase. Ma and Eaton[26] showed that dilute suspensions of wild-type and catalase-deficient *Escherichia coli* have identical sensitivities to H_2O_2, putatively because even wild-type catalase-positive *E. coli* organisms cannot maintain an internal:external concentration gradient of this highly diffusible oxidant. However, concentrated suspensions or colonies of catalase-positive *E. coli* did preferentially survive H_2O_2 challenge, and could even crossprotect adjacent catalase-deficient organisms. While this type of phenomenon must be considered, it is not directly relevant to this review because the physicochemical nature of the media discussed above is not compatible with the growth of individual cells at the time of inoculation.

Little is known about the nature of the secreted substances involved in the inoculum-dependent lag phase. Some substances known to be secreted fit our definition of autocrine substances. Siderophores (iron-transport compounds) have been shown to act as growth factors influencing the inoculum-dependent lag in *Bacillus* cultures[22]. Normally, the effect of siderophores is most pronounced in iron-deficient conditions[27], but as iron-containing molecules have a catalase-like effect, a role in the cooperative removal of H_2O_2 cannot be excluded. Recently, Cooper *et al.* reported a 'dramatic reduction' of the lag phase during the regrowth of *Nitrosomonas europea* starved for up to 6 weeks after the addition of N-(3-oxohexanoyl)homoserine lactone (OHHL) to the growth medium[28], a compound known among other things as an autoinducer of luminescence in *Vibrio fischeri* and of antibiotic production in *Erwinia carotovora*[8].

As with starved bacteria, cells of *Bacillus subtilis* grown from a small inoculum in poor media make several divisions before ceasing growth, unless siderophores are added; a culture with <100 cells per flask fails to initiate growth[22]. Cells of *Francisella* (formerly *Pasteurella*) *tularensis* cannot initiate growth to high optical density from an inoculum of <10^5 cells per flask, but such growth can be promoted by supernatant taken from the same growing culture[29].

In summary, the phenomenon of 'limited divisions' is actually rather common in microbiological practice. However, because the threshold for optical detection of growth is typically 10^6–10^7ml^{-1}, it has not been noticed in many cases.

Growth in the chemostat
Spirillum serpens grown in a chemostat in a poor and defined minimal medium with a low concentration of limiting substrate has a markedly lower steady-state cell concentration than that theoretically predicted. The existence of a threshold in cell concentration (approximately 10^5ml^{-1}) below which cells cannot grow led to the conclusion that there are 'growth-stimulating interrelationships between cells of the bacterial population in a medium which does not meet all the requirements for growth of the single cell'[30]. Interestingly,

these observations were made at concentrations of growth-limiting substrate greater than the K_m.

'Microendocrinology'
If bacteria can communicate using a chemical language, does this bacterial language contain the same 'words' (molecules) as that of higher organisms? Many studies (Table 1) have revealed the presence of vertebrate-hormone-like substances in bacteria (including steroid and polypeptide hormones, such as insulin), which are found to be specifically active towards mammalian cells. Specific high-affinity binding proteins for many hormones are found in bacterial cells (for reviews, see Refs 31,32); most recently, even the pheromone-binding lipocalins have been found in prokaryotes[33]. The possible similarities between signal-transducing systems in prokaryotes and eukaryotes have also been discussed[34–36], and Lyte in particular[37,38] has developed the idea that the growth of pathogens *in vivo* depends strongly on host-derived hormonal signals. Importantly, when the activity of the appropriate hormone or cytokine was tested in all these studies (Table 1), the effect on bacterial growth rate (usually in the exponential phase) was not at all pronounced, which is probably responsible for the apparent difficulties in reproducing some of these results (see Refs 39,40). Furthermore, in most of these experiments, the conditions used were actually those least suitable for detecting a stimulatory effect, that is, they used rich media and relatively large inocula, and they assessed the effect during the log phase. Lag-phase studies (discussed previously) indicate that the use of large inocula (leading to the carry-over of stationary-phase supernatant) and rich media can mask the stimulatory effect of growth factors. Lyte and Ernst found that catecholamines have a detectable (and, as judged by colony-forming units, substantial) effect on bacterial growth only if the starting concentration of cells is low[41,42].

Similarities with nucleated cells
Behaviour similar to that described above for bacterial cells also occurs in higher organisms. Unicellular organisms (ciliates) and cells in tissue culture have been shown to need factors synthesized by cells to multiply further. If cells of *Tetrahymena* are inoculated into defined medium at a concentration of <750 cells ml^{-1}, they cannot grow at all unless supernatant from growing culture or various 'inducers', including lipids, alcohols and porphyrins, are added (for reviews, see Refs 43,44). Somewhat related phenomena have been observed in yeast[45]. The 'growth factor' for another ciliate, *Paramecium*, has been isolated as a 17 kDa protein[46]. Recently, it was found that a ciliate peptide mating pheromone actually has growth-stimulation activity just like an autocrine growth factor[47]. Interestingly, interleukin 2 inhibits this mating activity[48].

Cultivation of previously starved fibroblasts in normal medium results in a low cell concentration, irrespective of seeding densities. Lens cells die when they are held in culture with a low initial concentration of cells, while conditioned medium from high-density cultures promotes their survival[49]. It has been proposed

Table 1. Some autocrine (pheromone) substances and animal hormones that stimulate the growth of bacteria and other unicellular organisms[a]

Organism	Role	Chemical nature	Refs
Bacillus	Reduction of lag phase, permit growth from small inoculum	Siderophores[b]	22
Escherichia coli	Stimulation of growth	IL-1, GM-CSF	62–64
Gram-negative bacteria	Stimulation of growth	Catecholamines	41,42
Micrococcus luteus	Resuscitation and stimulation of growth after dormancy	Unknown, protein	19[c]
Mycobacterium avium	Stimulation of growth	IL-6	65
Mycobacterium tuberculosis	Stimulation of growth	TGF-β1	55
Neurospora crassa	Stimulation of growth	Insulin	66
Nitrosomonas europea	Reduction of lag phase	N-(3-oxohexanoyl)homoserine lactone	28
Saccharomyces cerevisiae	Stimulation of growth	Insulin	67
Francisella (Pasteurella) tularensis	Permit growth from small inoculum	Not known, low molecular mass	23,29
Streptococcus (Enterococcus) faecalis	Stimulation of growth	Serotonin	68
Xanthomonas maltophila	Stimulation of growth	Chorionic-gonadotropin-like ligand, protein of 48 kDa	69,70
Tetrahymena	Permit growth from small inoculum	Lipids, alcohols, porphyrins, insulin, bovine serum albumin	43,44
Paramecium tetraurelia	Stimulation of growth	Protein of 17 kDa	46
Euplotes raikovi	Growth promotion, mating factor	Polypeptides	47

[a]Abbreviations: IL-1, interleukin 1; GM-CSF, granulocyte–macrophage colony-stimulating factor; TGF-β1, transforming growth factor β1.
[b]Siderophores may be nutrients rather than cytokines when iron is bound.
[c]Also this article.

that there is a universal mechanism for cell death, as in the above two cases, based on the idea that there may have been an absence or insufficient secretion of one or more factors that normally prevent growing cells from initiating apoptotic death[50]. A similar mechanism probably works in Tetrahymena[44]. Although a very different kind of programmed cell death has been described recently in bacteria[51,52], it is not known whether cell–cell communication preventing apoptotic death of the eukaryotic type occurs in prokaryotes.

Perspectives for bacterial pathogenicity
A developing bacterial culture might be considered to be a socially organized system in which cells are talking

Questions for future research

• Are the so-called 'uncultured microorganisms' dead or do they merely need some unknown and possibly autocrine growth factors for their cultivation in vitro? Were such factors produced and inactivated (or did they diffuse away) during the period of starvation in nature?
• Is there a critical cell concentration of viable bacterial cells in an inoculum below which cells cannot grow? Under what conditions and in what media can this be observed?
• Do mechanisms of apoptotic death similar to those of higher eukaryotic cells exist for bacteria?
• Could the growth of bacteria in poor (synthetic) media be improved using appropriate non-nutrient growth factors?
• How commonly does the development of bacterial infection depend on the hormonal (and cytokine) levels of the host (in the same way that melioidosis is connected with the insulin level)?

to each other using specific factors in many processes[10,32], clearly including multiplication. For unstressed (uninjured) bacteria and optimal growth media, the 'self-promoting' mode of culture growth can be masked owing to the high rate of production of growth factors and the sensitivity of the cells to pheromones [the 'Paramecium factor' acts at a concentration of ng ml^{-1} (Ref. 46)], which can result in the successful outgrowth from one bacterium in a test tube or to form a colony on an agar plate. Only under unfavourable conditions (poor medium, low cell concentration in the inoculum, starved cells or a combination of these factors) can this behaviour be observed. From this point of view, bacterial culture resembles tissue-cell culture, in which the requirements for growth factors are stronger and better known. (As an extreme, a bacterial colony might be considered to be an organism composed of physiologically distinct tissues[53].) Another case in which the self-promoting mode occurs (especially for cells that do not tend to divide completely, such as staphylococci) could involve so-called 'juxtacrine signals', where a membrane-bound growth factor on one cell interacts directly with its receptor on the adjacent cell.

Teleologically, social behaviour during culture growth clearly helps to increase biomass as quickly as possible, and to respond rapidly to changing nutritional and other circumstances[10]. For much laboratory practice, in which appropriate media and large inocula are usually used, the significance of such behaviour is probably low. However, it is entirely plausible that so-called 'uncultured' microorganisms[54] need growth factors to grow in vitro.

The self-promoting mode of cell growth could have serious implications for medicine when the initial concentration of infecting bacteria is likely to be very low. For example, transforming growth factor β1 accelerates the growth of *Mycobacterium tuberculosis* in monocytes and might be important in the pathogenesis of tuberculosis[55], while the low insulin concentration in the blood of diabetics results in a significant proliferation of *Pseudomonas pseudomallei*, the causative agent of melioidosis[56]. Similarly, transferrin is essential for the growth and survival of *F. tularensis* in the acidic vacuole of mouse macrophages[57].

This effect could be particularly important for infections in which bacterial cells may persist in a host organism in latent or dormant states, in particular, *M. tuberculosis*, which can become dormant in the tissues of humans and experimental animals, leading to latent disease. This idea is strongly supported by the transition of viable *M. tuberculosis* cells to non-replicating cells under microaerophilic conditions[58]. Domingue *et al.*[59] have found bacterial 16 S rRNA genes characteristic of Gram-negative bacteria in biopsies of patients with interstitial cystitis, although routine cultures of bacteria from patients' urine were negative, and persistence of bacteria in a dormant form has been suggested to be involved in the etiology of this disease[59] and others[60]. It is easy to suggest that specific signal(s), derived from the host or the invader, might help to activate dormant forms and to promote their growth; the effect on growth would be similar to results under laboratory conditions[19,28]. At present, this area of microbiology is totally undeveloped; a broader approach uniting bacterial physiology, biochemistry and medical microbiology is needed to tackle these questions[61].

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An integrin-like protein in *Candida albicans*: implications for pathogenesis

Margaret K. Hostetter

In vertebrates, members of the integrin family of hetero-dimeric transmembrane proteins are intimately involved in adhesion, morphogenesis and signaling. The recent discovery of an integrin-like protein in the primitive eukaryote *Candida albicans*, which participates in similar processes, raises provocative questions about candidal pathogenesis and larger evolutionary relationships. This brief review addresses key concepts in our current understanding of integrin function in both simple and complex eukaryotic cells, and forecasts some of the potentially exciting roles for integrins in microbial pathogenesis.

Candida albicans is the foremost fungal pathogen in neonates, diabetics and immunocompromised people. The discovery of integrin-like proteins in this yeast and the involvement of these proteins in adhesion, morphogenesis and signal transduction suggest important roles for primitive integrins in the pathogenesis of infectious diseases, and as paradigms for signaling and differentiation in vertebrate cells.

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Vertebrate integrins

Structure

The integrin-like protein of *C. albicans* is antigenically, structurally and functionally related to the α subunits of the β_2 integrins (α_M and α_X; also known as CD11b and CD11c, respectively); hence, these leukocyte adhesion glycoproteins can be considered as prototypes for the purposes of this discussion. The leukocyte adhesion glycoproteins are expressed as heterodimers composed of a single α subunit (α_L, α_M or α_X) and a common β subunit of 95 kDa. Four α subunit domains endow these proteins with considerable functional versatility (Fig. 1a). (1) Near the amino terminus, an I (inserted) domain of approximately 180 amino acids is essential for the recognition of ligands, such as fibrinogen and the C3 degradation fragment, iC3b. Many integrins recognize the tripeptide Arg-Gly-Asp (RGD) in their ligands in the extracellular matrix; however, α_M binds iC3b despite mutation of the RGD tripeptide[1,2]. (2) Ligand binding is facilitated by the presence of three linear divalent-cation-binding sites at the carboxy-terminal side of the I domain; these are conserved among all α subunits. In α_M and α_X, the binding of ligands, such as iC3b, requires physiological concentrations of calcium, although a conformationally dependent, internal divalent-cation-binding site within the I domain preferentially requires manganese[3]. (3) A hydrophobic transmembrane domain of 25–30 amino acids abuts a highly conserved cytoplasmic sequence, KxGFFKR (where x denotes any amino acid)[4–6]. The GFFKR motif binds calreticulin[7], and is thought to modulate the affinity of the α subunit for ligands. (4) The cytoplasmic tail links the extracellular portion of the integrin molecule to the cytoskeleton and contains a single tyrosine residue.

Adhesion, morphogenesis and signaling

The leukocyte integrins are required for the firm adhesion of leukocytes to the endothelium via intercellular adhesion molecule 1 (ICAM-1), and for subsequent extravasation and transendothelial migration. Other integrins are intimately involved in the morphogenesis of tissues as diverse as the developing lung or metastasizing melanoma[8,9].

Integrin-mediated signaling involves both 'outside–in' and 'inside–out' transmission. In 'outside–in' signaling, attachment of integrins to proteins in the extracellular matrix transduces a signal to the interior of the cell via the integrin transmembrane and cytoplasmic domains. Calcium flux, cytoskeletal rearrangement and regulation of gene expression through the activation of tyrosine-kinase pathways ensue[10]. 'Inside–out'