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Signals of Growth Regulation in Bacteria

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Summary

A fundamental characteristic of cells is their ability to regulate growth in response to changing environmental conditions. This review focuses on recent progress towards understanding the mechanisms by which bacterial growth is regulated. These phenomena include the “viable but not culturable” state (VBNC), in which bacterial growth becomes conditional; and “persistence”, which confers antibiotic resistance to a small fraction of bacteria in a population. Notably, at least one form of persistence appears to involve the generation of non-growing phenotypic variants after transition through stationary phase. The possible roles of toxin-antitoxin modules in growth control are explored, as well as other mechanisms including contact-dependent growth inhibition, which regulates cellular metabolism and growth through binding to an outer membrane protein receptor.

Introduction

This review focuses on recent advances in our understanding of bacterial growth regulation, with an emphasis on the mechanisms that control entry and exit from a slow growth or non-growth (dormant) state, excluding spore formation. This topic has relevance to a number of important aspects of bacterial biology including resistance of a small fraction of a bacterial population to killing by an antibiotic, termed “persistence”. The maintenance of bacterial viability without growth impacts human health in a number of ways including maintenance of pathogen reservoirs and chronic infections such as tuberculosis and melioidosis. This has been a difficult area of research, in part due to phenotypic variability in which only a small fraction of bacteria are within a dormant state in a population, making it hard to isolate and study dormant cells. Moreover, since many genes influence cell growth, it has been a challenge to identify those that constitute specific pathway(s) for dormancy/antibiotic resistance. Our aim in this review is to delineate some of the key findings and concepts in growth control, bringing together new developments in different fields of research that may impinge on one another.

The viable but not culturable (VBNC) state

Colwell and co-workers first reported that bacteria can fail to grow on laboratory media but still appear viable based on outer membrane integrity and the ability to recover growth through temperature shifts [1]. This phenomenon, termed “viable but not culturable” (VBNC), has now been described for over 50 bacterial species using various criteria for viability including propidium iodide exclusion, redox activity, and green fluorescent protein reporter expression;

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but of course none of these assays proves that cells are actually viable, only that they retain some function(s) of living cells. The word “viable” indicates that under some condition the VBNC bacteria must be able to resuscitate and grow, and thus it has been pointed out that VBNC is a misnomer [2]. Despite these nomenclature problems, it appears that bacteria can become nonculturable under certain conditions while maintaining at least some metabolic activity (i.e., not dormant) but can be recovered under other conditions [3]. This poses a problem for waterborne pathogen monitoring in estuarine and marine environments (such as *Vibrio* spp.) as pointed out by Colwell [1]. Although many papers have been published on the VBNC phenomenon, no specific mechanism has been identified. Indeed, because many environmental conditions induce the VBNC state in different bacterial species, it seems likely that there is no single underlying mechanism. A well-characterized VBNC system is in *Vibrio vulnificus*, in which prolonged incubation at 5°C leads to the VBNC state. Work from several groups suggests that hydrogen peroxide could play a role in VBNC because a fraction of VBNC cells grow on media containing antioxidants such as catalase. Although growth in the presence of catalase slowed the formation of VBNC cells, eventually virtually all cells became VBNC, indicating that sensitivity to oxidation is only one factor that limits growth [4]. Recent data from Abe *et al.* [5] support this hypothesis. Chemical mutagenesis of *V. vulnificus* yielded VBNC suppressor mutants that retain culturability after low temperature stress. One VBNC suppressor mutant expressed glutathione *S*-transferase to higher levels than wild type at low temperature and was found to be more resistant to hydrogen peroxide. However, this mutant almost certainly contains additional uncharacterized mutations, as indicated by its increased expression of CspA (cold-shock protein) homologues. In summary, the term VBNC likely encompasses several distinct mechanisms by which bacteria become unable to grow on certain media. Whether there is any commonality to bacterial VBNC mechanisms remains to be determined.

Contact-dependent growth inhibition

Recently a phenomenon called “contact-dependent growth inhibition” or CDI was described, in which cell growth is controlled by direct cell-to-cell contact mediated by the CdiA-CdiB two-partner secretion (TPS) system. CdiA-CdiB is present in certain *Escherichia coli* strains, and homologous proteins are found in many bacterial species [6]. By homology with other TPS systems, CdiB appears to be an outer membrane protein required for the transport and assembly of CdiA at the cell surface. The CDI receptor has been identified as BamA [7], also known as YaeT, which is an essential, highly conserved outer membrane protein required for the biogenesis of beta-barrel proteins in gram-negative bacteria [8]. Concomitant with a block in cell growth, CDI induces significant reductions in respiration, proton motive force, and ATP levels [9]. The mechanism that connects interaction with BamA at the cell surface to down-regulation of metabolism is unclear, but it may involve the inner membrane multidrug resistance protein AcrB since *acrB* mutants are resistant to CDI [7]. One possibility is that CdiA interacts with AcrB through BamA to modulate its activity. AcrB exploits the proton-motive force (pmf) to couple proton import to the export of small toxic molecules. Thus, CdiA could induce AcrB to open its proton channel and dissipate pmf. This speculative model for the CDI mechanism is outlined in Fig. 1A.

Another important aspect of the CDI mechanism involves an open reading frame that overlaps with *cdiA* and encodes a small protein termed CdiI that confers immunity to CDI [9]. Expression of CdiI in target cells is sufficient for protection from CDI⁺ inhibitor cells (Fig. 1A). CdiI is also necessary to protect CDI⁺ cells from inhibiting their own growth. By removing CdiI from the regulatory control of CdiBA, an autoinhibition system was developed in which CDI can be induced in a uniform population of cells. This system was used to demonstrate that CDI is a reversible process, at least under laboratory conditions. Autoinhibited cells resume growth within two hours after induction of the CdiI immunity protein. Recovery also requires

an energy source, which could re-establish the proton gradient via the electron transport chain. The proton gradient increases in recovering cells prior to respiration and cell division, suggesting that it plays a critical role in mediating CDI. CDI shares features with the VBNC phenomenon in that CDI-inhibited cells exclude propidium iodide, and their growth can be induced under specific conditions [9]. This raises questions about the function of CDI systems, which appear to be widespread amongst gram-negative species [9]. Because CDI-inhibited cells are metabolically down-regulated, CDI could be a counter-surveillance mechanism, allowing bacteria to hide-out within host tissues or the environment. Alternatively, CDI could be used as a bacterial warfare system to inhibit growth of neighboring cells, for example in a biofilm.

Other growth control mechanisms

Other interesting phenomena involving control of cell growth and metabolism have been recently described. An evolved variant of *E. coli* K-12 was shown to inhibit its ancestral form in stationary phase, through a contact-mediated process called stationary contact-dependent growth inhibition or SCDI [10]. In eight independent cultures the *glgC* gene involved in glycogen metabolism was affected, with resulting overexpression of glycogen. In parallel with CDI, there is an immunity component to SCDI but it is the mutated form of *glgC*! Thus, glycogen overexpression appears to induce SCDI and provides immunity to SCDI, by an unknown mechanism. An “identification of self” (*ids*) locus in *Proteus mirabilis* was recently described that regulates growth at boundaries formed between different swarming strains and is somehow able to recognize self from non-self •[11]. Although the *ids* mechanism is not known, it could involve cell-to-cell contact similar to CDI and SCDI. Other recent examples of growth regulation by contact have come from the study of bacterial symbiosis. The flagellum of *Pelotomaculum thermopropionicum* strain S1 mediates an interaction with *Methanothermobacter thermoautotrophicus* strain ΔH , allowing the two bacteria to cooperate metabolically. Of note, the FliD protein at the flagellar tip induces methanogenesis in *M. thermoautotrophicus* upon contact with the cell surface. The hydrogen gas generated during propionate fermentation from *P. thermopropionicum* is used by its partner for methane formation, conveniently keeping the partial pressure of H₂ low and thus facilitating continued propionate oxidation and growth •[12] (Fig. 1B).

In addition to cell contact-mediated growth control, soluble mediators may also regulate cell growth •[13-15]. Recent results indicate that released autoinducers may regulate the growth rate of *Vibrio harveyi* independent of the effect of quorum sensing on bioluminescence [13]. *Streptococcus oligofermentans* inhibits growth of *S. mutans* by generation of hydrogen peroxide •[15] a growth inhibitory compound that has been associated with the VBNC state (above). Mycobacteria including *M. tuberculosis* are known to have a dormant growth phase which is induced by cellular stresses including hypoxia. Serine/threonine protein kinases and two-component regulatory systems have been implicated in the control of mycobacterial cell division and growth, respectively. A recent provocative report indicates that *M. marinum* and *M. bovis* form spores in stationary phase •[16]. Spores are the ultimate dormant cell and could account for the chronic nature of tuberculosis if *M. tuberculosis* is shown to sporulate. There are almost certainly many undiscovered interactions occurring between bacterial species that regulate growth and metabolism; we are just beginning to scratch the surface.

Chromosomal TA modules as modulators of growth

Toxin-antitoxin modules (hereafter termed TA modules) are widely distributed, two-component systems implicated in bacterial growth control [17]. TA modules encode a stable “toxin” protein, whose activity results in either growth arrest or cell death, and an unstable “antitoxin” that counteracts toxin activity (Fig. 2A). Antitoxins are either antisense RNAs that

suppress toxin expression, or labile proteins that bind and inactivate their cognate toxin (for a comprehensive review of TA modules, see [17]). The antitoxins encoded by the latter proteic TA systems also act as transcriptional repressors that autoregulate TA expression. TA systems were first described as addiction modules that contribute to plasmid maintenance. In plasmid-free daughter cells, cytoplasmically inherited toxin molecules are activated by antitoxin degradation. Because plasmid-free cells no longer carry the TA operon, degraded antitoxin cannot be replenished and the unopposed toxin arrests growth in a process termed “post-segregational killing”. Thus, plasmid-encoded TA systems help suppress the proliferation of plasmid-free cells. Intriguingly, TA modules are also commonly found on eubacterial and archaeal chromosomes, with some species containing dozens of the gene pairs [18,19]. The physiological function of chromosomal TA modules has been the subject of much speculation and debate over the past decade [20,21]. Indeed, evidence of extensive horizontal TA module transfer has raised the possibility that these genes are merely selfish genetic elements that confer no benefit to host cells [19,20]. However, chromosomal TA systems can stabilize their genomic neighborhood [22] and act as “antiaddiction” modules to prevent post-segregational killing mediated by related plasmid-encoded toxins [23]. Additionally, the *E. coli mazEF* TA module is proposed to induce programmed cell death (PCD), which requires cell-to-cell communication through an extracellular signaling peptide [24,25]. Although a MazF homolog has recently been shown to be required for PCD during fruiting body development in *Myxococcus xanthus* [26], PCD in *E. coli* has not been replicated by other groups and remains controversial [27,28].

Chromosomal TA modules have also been proposed to function as regulators of cell growth in response to environmental stress (Fig. 2B). In *E. coli*, Lon- and ClpAP-mediated antitoxin degradation is induced by starvation and other conditions that inhibit transcription and translation [29-31]. In most cases, the liberated toxins are “mRNA interferases” (RNases that preferentially cleave mRNA), which inhibit protein synthesis and rapidly arrest growth. Gerdes and colleagues have shown that mRNA interferases elicit a bacteriostatic state that is readily reversed by cognate antitoxin expression [27,29,30]. These results appear inconsistent with the PCD model, and suggest that TA modules induce growth arrest rather than cell death. According to this model, environmental stress results in transient toxin activation, which rapidly shuts down protein synthesis until more hospitable conditions return. Because TA transcription is de-repressed by antitoxin degradation, the cell is poised to produce more antitoxin to counteract toxin activity after the cell has adapted to the stress. Recovery from mRNA interferase activation is also facilitated by the tmRNA quality control system, which recycles ribosomes stalled during translation of toxin-cleaved mRNAs [29,30,32]. A recent direct test of the stress-response hypothesis found that TA modules confer no recovery advantage to *E. coli* cells after a variety of environmental stresses [28]. However, additional TA modules have subsequently been identified in *E. coli*, and indirect evidence from other species appears to support the growth control model. The *fitAB* (fast intracellular trafficker) locus of *Neisseria gonorrhoeae* is homologous to the *vapBC* family of TA modules [33,34], and is predicted to encode an mRNA interferase toxin. Disruption of *fitAB* has no effect on host-cell independent growth, but significantly increases the rate at which *N. gonorrhoeae* invades and replicates within epithelial cells [33]. Similarly, disruption of the *ntrPR* TA module from *Sinorhizobium meliloti* produces mutants that form root nodules with leguminous plants more efficiently, and fix more nitrogen than wild-type cells [35]. Transcriptional arrays also suggest that *ntrR* mutants have increased capacity for transcription and translation under conditions that mimic symbiosis [36]. Thus, these TA modules appear to limit bacterial growth upon encountering eukaryotic host cells, which may be triggered by specific environmental signals.

Persistence, a mechanism for growth control by phenotypic variation

A phenomenon called “persistence” was previously described in which addition of penicillin to cultures of *Staphylococci* killed most of the cells, but rare “persister” cells survived. Persistence is not a genetically heritable trait, and thus differs from the many mechanisms known by which bacteria can become resistant to antibiotics [37,38]. The low frequency and non-heritable characteristics of persister cells have made it difficult to isolate pure populations for study, and thus progress in this area towards understanding mechanism has been slow. Recently, the Balaban group has gained insight into one class of persister cell termed type I •• [39,40]. The formation of these persister cells is linked to the HipA-HipB TA system, since a mutation in *hipA(7)* increases the frequency of persisters by ~1,000-fold [41]. Type I persisters are generated in stationary phase and are non-dividing, dormant cells that spontaneously arise in the population. Using a microfluidic device that allows examination of individual bacterial cell lineages, Gefen *et al.* recently showed that although type I persister cells arise in stationary phase, they do not enter the dormant state until about 1.5 h after exiting stationary phase •• [39]. This suggests that the dormancy program is initiated by signals in stationary phase (e.g. (p)ppGpp [42]), but its implementation is delayed. Notably, type I persister dormancy also temporarily confers resistance to phage λ by blocking the lytic gene expression program, which can resume following exit from the persister phase [43]. A different type of persister cell, denoted class II, arises during logarithmic growth, and exhibits an ~10-fold slower growth rate. The uncharacterized *hipQ* mutation increases the appearance of type II persisters and has been used for analysis. Notably, unlike type I persisters, type II cells continue to grow slowly in the presence of ampicillin for a few generations [40] suggesting that the type II persister state may be weakly heritable. This phenomenon could be mediated by DNA methylation, which is heritable, but can be lost within a couple generations [44].

The underlying mechanisms of type I and type II persistence are unclear. Moreover, these persister cell types have been obtained using mutant bacteria and thus it is not clear how they relate to persisters arising in a wild-type cell population. Based on analysis of semi-quiescent wild-type *E. coli* from logarithmic phase cells, certain TA modules are up-regulated •[45], suggesting that TA modules may contribute to persistence. However, because persisters (i.e. type I) are characterized by negligible macromolecular synthesis, it is equally possible that TA activation is a result, rather than a cause, of persistence. It has been suggested that persistence could be the result of cellular aging [46], based in part on the observation that although *E. coli* appears to undergo symmetric division based on cell morphology, cells that inherit the older cell pole have slower growth rates and eventually senesce [47]. Thus, older and slower growing cells in the population could be persister cells. However, this hypothesis seems inconsistent with the observation that type I persister cells do not become dormant until 1.5 h following exit from stationary phase. Another possibility is that persistence is regulated by a bistable switch controlled by a stochastic process [48]. This is similar to the control of competence in *Bacillus subtilis*, in which expression of the positively autoregulated ComK competence factor occurs in only about one-fifth of cells due to a stochastic switch influenced by cellular “noise” •[49]. It has been suggested that persistence may be controlled by a double-negative bistable switch in which an increase in the ratio of toxin to antitoxin could occur in a cell due to a stochastically higher level of degradation of antitoxin, leading to a decrease in translation in that cell [50]. This should result in a further decrease in translation due to the higher stability of toxin compared to antitoxin.

Conclusions

Identification of the signaling mechanisms that regulate cellular growth is critical to understand how microbes colonize diverse environments. Bacterial generation times are generally short, allowing them to play a bet-hedging strategy in which phenotypic variation is generated by

mutation. Many of these mechanisms are well understood due in large part to the ease with which mutants can be isolated. In addition, a number of epigenetic phenomena have been identified, including the VBNC and persistence states, by which bacteria create phenotypic diversity. Most of these phenotypes are non-heritable, in some cases not well defined, and in virtually all instances poorly understood at the mechanistic level. CDI provides a means by which VBNC cells can be generated and is amenable to mechanistic studies [9]. A detailed understanding of some chromosomal TA systems has been achieved, but solid evidence for a direct role in cellular growth control and persistence is lacking. The recent development of new methods of analysis •[38,39] and isolation •[45] of slow-growing cells from a large population should increase the pace of discovery of non-heritable growth regulatory mechanisms.

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A. Contact-dependent metabolic and growth suppression

B. Contact-dependent metabolic induction

Figure. 1. Examples of contact-dependent alteration in metabolism and growth

(A). *Escherichia coli* containing the *cdiBAI* genes express CdiB, which is predicted to reside in the outer membrane and function in export of CdiA to the cell surface. CdiA is postulated to mediate binding to the BamA receptor in the outer membrane (OM) of target cells, and reduce the proton gradient, respiration, ATP, and cell growth/division of target cells. CdiI provides immunity to autoinhibition by cognate CdiA effector. The CDI mechanism may involve interaction with the multidrug efflux pump AcrB located in the inner membrane (IM) [6,7,9].

(B). *Pelotomaculum thermopropionicum* strain S1 expresses flagella that bind to its syntrophic partner *Methanothermobacter thermoautotrophicus* strain ΔH , keeping the cells close by tethering. Binding of the FliD cap protein of the flagellum to an unidentified receptor on *M. thermoautotrophicus* alters cellular metabolism including induction of methane formation. Hydrogen gas from *P. thermopropionicum* is used for methane production, and serving to keep

H₂ levels low and enabling continued fermentation of propionate by *P. thermopropionicum* • [12].

(A) TA module structure and activity **(B) Stress-response regulator hypothesis**

Figure 2. Toxin-antitoxin (TA) module organization and the stress-response regulator hypothesis
(A) TA module genetic organization. TA systems are typically two-gene operons with the antitoxin encoded upstream of the toxin gene. Proteic antitoxins bind their cognate toxins and inhibit toxin activity. Antitoxins and toxin-antitoxin complexes repress TA module transcription. In *Escherichia coli*, toxin activation involves degradation of antitoxin by either the Lon or ClpAP proteases. Activated toxins inhibit cell growth and/or lead to cell death.
(B) The stress-response regulator hypothesis of chromosomal TA function. Environmental stresses such as starvation activate chromosomal TA modules through antitoxin degradation. Many toxins are mRNA-specific RNases that rapidly inhibit protein synthesis, inducing a bacteriostatic state. Upon adaptation (or removal of the stress), antitoxin synthesis resumes to counteract toxin activity, and tmRNA activity rescues ribosomes arrested on toxin-cleaved messages.