CHAPTER 1

Physiological and molecular aspects of growth, non-growth, culturability and viability in bacteria

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INTRODUCTION

Infection requires growth of pathogens in host tissues or on host epithelia. Cessation of growth is generally correlated with control of infection. Clinically latent infections may reflect microbial growth balanced by host control mechanisms such that the interaction remains below the threshold of detection. Alternatively, the pathogen may have genuinely ceased growth and survive in some form of stasis. In most cases we cannot distinguish between these possibilities. However, there have been important recent advances in our understanding of bacterial populations in which net growth cannot be detected and in recognising the limitations of in vitro culture as a means of determining the presence and viability of bacteria. These advances present new opportunities to study the role of non-growing and dormant bacteria in infection and to consider the degree to which culture-based methods may give a false impression of the absence of pathogens during infection, clinical latency and treatment.

The progress of molecular methods in microbiology challenges us to determine the molecular basis of growth and its regulation and to develop such methods to detect growth and viability. In the present context, the long-term aim must be to recognise growth states of microbial populations in the human host.

BACTERIAL GROWTH

Growth involves the accumulation of biomass and may include genomic replication, cell division and an increase in the number of propagules of the organism concerned. For most bacteria it is generally held that, after division,
a newly formed cell placed in an environment favourable to growth will double its mass then divide to form two equal-sized progeny via binary fission. This process has been subjected to detailed analysis and is discussed from a highly selective viewpoint here. For more comprehensive and introductory discussions, the reader is referred to recent reviews (35, 55, 57, 66, 82, 104).

Our current understanding of bacterial growth derives overwhelmingly from studying selected organisms in broth cultures. Liquid cultures are convenient; most variables can be precisely controlled, and the scale can be adjusted to provide sufficient biomass for almost any form of analysis. In achieving reproducible results between laboratories, the development of chemically defined media, consistent inocula and the recognition of growth states that can be detected by sequential optical density or turbidity measurements have provided a platform for further development. The widely accepted terminology of lag, exponential (or log) and stationary phases of growth in batch culture provides essential physiological points of reference and these are often applied, with scant justification, to bacterial cells and populations outside the highly defined laboratory environments indicated.

A detailed analysis of the energetics and stoichiometry of bacterial growth has been made possible by analysing bacterial populations growing at constant rates in chemostat or turbidostat cultures (35, 57, 93). These systems provide a relatively reproducible gold standard in which a state referred to as “balanced exponential growth” can be achieved for extended times. The resultant population of cells is generally believed to be uniform and growing at similar rates. Thus it is considered legitimate that analyses of cells in balanced exponential growth can be divided equally amongst all the cells present in the sample to yield estimates of content or activity per cell present.

An important alternative approach has been to start by considering the bacterial cell cycle, which starts with the birth of a cell by binary fission of a parental cell and ends with the division of the new cell. This kind of work draws substantially on our understanding of the eukaryotic cell cycle, where the biochemical and physiological events have been separated into distinct phases (G1, S, G2, and M with or without G0), and has been pursued using techniques that provide large populations of cells that are all at the same stage of the cycle. While some controversy continues, it is generally thought that events that are considered critical for progression through the cell cycle in eukaryotes (e.g., initiation and termination of DNA synthesis) are not similarly regulated in bacteria. Rather, the short-term fate of a cell is determined by the rate at which it accumulates biomass and by the particular size-growth
rate ratios at which division is initiated (18). Recently, however, Walker and colleagues (120) have suggested that the $umuDC$ component of the bacterial SOS response functions in a manner analogous to the eukaryotic S phase checkpoint. The analogy is complicated by the fact that rapidly growing bacteria initiate new rounds of chromosome synthesis before the last has finished. The authors also point out that the associated checkpoint and DNA repair systems are well suited to dealing with DNA damage accumulated during stationary phase at the time of re-entry into the growth cycle (92).

Most biochemical knowledge obtained with these methods refers to large cell populations ($\sim 10^7$) of readily culturable bacteria in exponential growth phase. Here, we are primarily concerned with the behaviour of pathogens during infections. Not only will these organisms rarely be in a simple suspension phase but also it seems most unlikely that the environment will be conducive to unimpeded exponential growth. Evidently, the degree to which most of our knowledge of bacterial growth is applicable to the environments that primarily concern us must be limited.

Laboratory studies on bacterial growth have also provided limited information regarding growth in colonies on or in solidified laboratory media (74, 125) and in biofilms (37, 75). While information on the growth of bacteria in colonies and in broth may be valuable in designing isolation and culture media for medically important bacteria (35), growth in biofilms is probably a principal mode of bacterial propagation in natural communities. In infections involving fluid-filled spaces (e.g., cystitis) it is plausible that the growth phases recognised in broth culture may be applicable and the relevance of biofilm growth to colonization of intravascular devices also seems certain. However, beyond these examples, assignment of in vitro-defined growth phases to pathogens at various stages in infection is largely speculative.

Molecular Information Related to Bacterial Growth

Studies on carefully defined broth cultures remain the principal reliable source of information on the molecular basis of bacterial growth. As key genes involved in growth and its regulation have been identified through recent pre- and post-genomic studies, the possibility of determining the importance of these genes to infection through deletion, over-expression and reporter studies has been extensively exploited. In the context of infection, it is conspicuous that technologies applied to detection of genes essential for growth in vivo, such as signature tagged mutagenesis, have often detected genes that appear integral to growth and metabolism (as opposed to classical
aspects of virulence such as invasion and toxicity) as essential for in vivo survival (e.g., 63, 94).

A somewhat arbitrary selection of genes whose expression has been related to growth in various ways is reviewed below. Ultimately it should be possible to recognise all the genes that are required for growth in specific environments. It seems likely that these will fall into two categories: those required in all circumstances and those required only for special environments.

Ribosomal RNA

A single E. coli chromosome generally carries seven copies of the genes encoding ribosomal RNA. In contrast, the Mycobacterium tuberculosis chromosome encodes only one copy. Given the greater than tenfold difference in minimum doubling times between these organisms (0.3 h vs. 6 h), it seems likely that this is no accident. The 16S, 23S and 5S genes (and some tRNA genes) are located in tandem and are initially transcribed into RNA as a single molecule, which therefore includes the so-called intergenic transcribed sequences (ITSs). The transcript is then processed into the recognised subunit components, and these combine with ribosomal protein to form functional ribosomes. Aside from the central role now occupied by the 16S molecule and the ITSs in the classifications of Bacteria and Archaea, the rate at which these genes are transcribed and the 165rRNA content of bacterial cells has been directly correlated with bacterial growth rates in vitro (14, 20). Analysis of these genes and their products in samples therefore presents opportunities to both identify and make some inferences about the protein synthetic capacity and growth rate(s) of the organisms present.

Chromosome Replication

Chromosome replication requires more time to complete than the time available between cell divisions during rapid growth of E. coli. The organism circumvents the potential problem of producing cells with less than a single complete genome by initiating rounds of chromosome replication at intervals compatible with the cell replication rate. Initiation always starts at the same locus (oriC) and proceeds bi-directionally to the terminus region (76). One consequence of this is that cells in rapidly growing populations contain more than one chromosome replication fork in progress, and the largest cells present (i.e., those close to fission) have a chromosomal DNA content in excess of two copies of the complete genome. The mechanism by which the interval between initiating rounds of chromosomal replication is regulated is not understood, but several gene products are known to be essential. Amongst these the DnaA protein, a DNA-, ATP- and ADP-binding
protein, has been most extensively studied and appears to play a central role in assembly of the initiation complex (76). A further consequence of the pattern of replication is suppression of transcription of specific genes as the replication fork passes through. This leads to apparent cell-cycle-related gene regulation in synchronised cultures (129).

**Cell Division**

Understanding of the molecular basis of bacterial cell division has advanced dramatically over recent years. Progress has been fuelled by development of immunocytochemical techniques for bacteriology and by the use of translational reporter fusions with the green fluorescent protein. These developments have enabled localisation of key molecules that determine the site and process of cell division. Amongst these the tubulin-like molecule FtsZ has been extensively studied. Around 10,000 molecules of this key protein are present in each *E. coli* cell and, like its eukaryotic counterpart, it is present in both soluble and polymerised forms. Location of FtsZ polymers in ring structures indicates the site of prospective septum formation, and using *ftsZ::gfp* translational fusions, it has been possible to observe, in real time, the formation and subsequent contraction of the FtsZ ring in parallel with septum formation and cell division (67, 118). Although FtsZ possesses GTPase activity, it is not known whether it provides the physical force required for septation and fission. Inhibition of FtsZ polymerisation by SulA (a protein produced as part of the SOS response) in growing cells lead to filamentation, thereby illustrating the key role of FtsZ in fission. All bacteria so far studied possess FtsZ homologues, and the relative abundance of the molecule makes it an attractive target for study in clinical samples. The presence of FtsZ rings indicates active cell division, and in *Bacillus subtilis*, asymmetric positioning of the ring indicates the onset of sporulation (64).

**Global Regulatory Proteins**

These molecules direct differential gene expression by binding either to DNA or to components of the transcription/translation apparatus. Their own levels of expression and activity are modulated by a variety of internal and external stimuli. It would be impractical to discuss even a small minority of these molecules here, but the levels and/or activities of some prominent examples in relation to bacterial growth are outlined in Table 1.1. A full discussion of these molecules is in Chapter 2.

A discussion of the complex regulatory hierarchy and network that are emerging from the study of these proteins and their cognate regulons is beyond the scope of this chapter. The painstaking process of analysing their
<table>
<thead>
<tr>
<th>Category</th>
<th>Protein</th>
<th>Gene(s)</th>
<th>Some relationships to growth</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Regulation via DNA topology</strong></td>
<td>H-NS</td>
<td>osmZ</td>
<td>Levels in constant ratio to DNA content during growth. Depressed in stationary phase</td>
<td>Histone-like DNA binding protein that represses transcription of multiple genes</td>
</tr>
<tr>
<td></td>
<td>LRP</td>
<td>lrp</td>
<td>Repressed by growth in rich medium</td>
<td>Selective repression and activation of genes appropriate to available nutrient sources</td>
</tr>
<tr>
<td></td>
<td>IHF</td>
<td>ihfA</td>
<td>Induction on entry into stationary phase; expression dependent on ppGpp</td>
<td>Interaction with DNA induces 180° bend enabling long-range interactions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ihfB</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SOS response</strong></td>
<td>RecA</td>
<td>recA</td>
<td>Activation of RecA by DNA damage induces cleavage of LexA and de-repression of SOS genes and arrest of cell division.</td>
<td>RecA controlled genes effect DNA repair and maintain λ-like phage lysogeny.</td>
</tr>
<tr>
<td></td>
<td>LexA</td>
<td>lexA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Alternate σ factors</strong></td>
<td>σ^{70}</td>
<td>rpoD</td>
<td>σ^5 levels increase on entry into stationary phase and on sudden growth arrest.</td>
<td>Main RNA polymerase σ subunit Heat shock induced</td>
</tr>
<tr>
<td>(required for transcription)</td>
<td>σ^{38}</td>
<td>rpoS</td>
<td></td>
<td>Stationary phase and stress induced</td>
</tr>
<tr>
<td></td>
<td>σ^{32}</td>
<td>rpoH</td>
<td>Alternate σ factors appear to compete with σ^{30} for binding to a limited amount of core RNA polymerase. Promoter specificity is modulated by alternate σ factor binding</td>
<td>Induced by extreme heat shock and regulates extracellular proteins</td>
</tr>
<tr>
<td></td>
<td>σ^{24}</td>
<td>rpoE</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>σ^{28}</td>
<td>fliA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>σ^{54}</td>
<td>rpoN(glnF)</td>
<td></td>
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Excerpt
More information
in combination with many other factors (e.g., H–NS, LRP, IHF & ppGpp). In *B. subtilis* a succession of alternate σ factors directs the programme of gene expression in sporulation.

Control of nitrogen metabolism

<table>
<thead>
<tr>
<th>Universal stress response proteins</th>
<th>UspA</th>
<th>.uspA</th>
<th>Induced in late exponential phase and by all known stress responses (σ^70 and ppGpp dependent)</th>
<th>Regulation via phosphorylation of target proteins?</th>
</tr>
</thead>
<tbody>
<tr>
<td>UspB</td>
<td>.uspB</td>
<td>Induced during transition phase (σ^38 and ppGpp dependent)</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>General metabolic regulators</td>
<td>CRP</td>
<td>.crp</td>
<td>Low glucose (e.g., on entry into stationary phase) leads to increased cAMP levels.</td>
<td>CRP–cAMP complexes activate or repress specific genes.</td>
</tr>
<tr>
<td>FNR</td>
<td>.nirA</td>
<td>FNR senses oxygen (anaerobic/aerobic growth)</td>
<td>Reduced FNR activates or represses specific genes related to anaerobiosis.</td>
<td></td>
</tr>
<tr>
<td>ArcA</td>
<td>.arcA</td>
<td>ArcA phosphorylated by cell membrane–associated ArcB quinone-responsive sensor kinase</td>
<td>Regulates catabolism of metabolic reserves</td>
<td></td>
</tr>
</tbody>
</table>

Note: Information presented predominantly relates to studies on *E. coli*. Abbreviations: IHF – integration host factor; USP – universal stress protein; CRP – cAMP receptor protein.

General sources: 78, 82, 83, 106. Specific sources: 1(3, 13, 24, 69); 2(120); 3(27, 29, 40, 64, 98, 109); 4(30, 33, 88); 5(11, 31, 34, 52, 62, 87, 105, 107).
respective roles is really only in its early stages and doubtless there are many regulators yet to be recognised and more functions to be defined. The relationships of these molecules to growth suggested in Table 1.1 emerge essentially from studies on samples from populations in specific growth phases during growth in defined media. With a few notable exceptions, information (e.g., 68) on the expression of regulatory proteins in contexts relevant to infection is very limited.

It should not be forgotten that there are many other classes of molecule that regulate bacterial phenotype. The underlying point here is that phenotype and growth state cannot necessarily be inferred from the detection of selective mRNA profiles. At the macromolecular level selective proteases (38) and antisense RNAs (121) have received much attention. Small molecules such as cyclic AMP (cAMP) (11) and guanosine tetraphosphate (ppGpp) (17, 43) are also recognised to have important regulatory roles. Many gene products affect their intracellular levels, and they have pleotropic allosteric effects on their respective binding proteins. The role of ppGpp, the key product of the stringent response, deserves special mention here since, by binding to the B subunit of DNA-dependent RNA polymerase, it provides another means of directing selective gene expression. The stringent response is stimulated by amino acid starvation and is generally associated with growth arrest (17).

In this selective survey the obviously important areas of energy metabolism, cell envelope biosynthesis and assembly and the so-called housekeeping genes have largely been ignored. However, the process of relating the expression of genes to bacterial growth could be extended to cover the entire genome and this can serve little function until we have an adequate interpretive framework. The global approaches offered by proteomics and arrays provide realistic prospects that this will be achieved.

GROWTH AND STASIS

The growth phases of bacteria in batch culture have been reviewed extensively elsewhere (35, 82, 93). Here the focus will be on individual cells and the populations they comprise. The aim is to introduce a framework within which cells in physiological states of particular significance to infection can be recognised and to cross-reference this to the classical growth phases.

Figure 1.1 presents a diagram outlining the various physiological states that can be recognised in relation to the growth of bacteria. Laboratory cultures can be observed at the population or cellular level and a comparison between these is attempted in Fig. 1.1. A central dichotomy is suggested between cells growing or committed to grow and those in some form of non-growing
Figure 1.1. Diagram comparing recognised states of growth and stasis in single cells and populations of bacteria. Point A identifies a cell committed to growth and replication; B, the initiation of growth; C, accumulated biomass below that required to initiate septation; D, septation prior to fission. E represents the point at which cells are notionally committed either to continued growth (equivalent to B) or to stasis. The conditions required for commitment to either pathway are only recognised at the population level. G represents cells that are not accumulating biomass, and F represents cells that may be formally described as dormant.

State (stasis). All the states possess potential for further growth and replication, and the cells concerned should therefore be considered viable.

Exponential Phase

In state A, a hypothetical cell, committed to growth and with appropriate resources available but not yet detectably growing, is envisaged. This cell may...
be adapting to a new environment or recovering from injury. Eventually the cell achieves state B where its phenotype is adapted to commence growth in its current environment and is seen as equivalent to the product of division in a growing culture. This cell grows as indicated through state C, where septum formation is initiated, to state D, where the septum is completed and fission is in progress. The separation of the progeny (E) into growing and static cells is arbitrary and serves only to illustrate alternate pathways. If conditions were conducive to continued growth then both progeny would be expected to continue in exponential growth.

The period between A and B is tentatively referred to here as the “specific lag phase.” In operational terms, the lag phase is measured as the interval between inoculation and the onset of detectable growth and can include an initial period of cell death and growth below the limit of detection. This period may include the times indicated between G and A or F and A, i.e., the time taken for non-growing cells to adapt and become committed to growth (see below).

An enormous amount of knowledge has been gained about populations dominated by cells in the exponential phase of the growth cycle indicated in Fig. 1.1. The rapidity of biomass accumulation is potentially breathtaking with doubling times of less than 30 minutes readily achievable by many pathogens that cause acute infection. It seems likely that such growth rates could underpin the rapid development of some infective conditions. The gradient of the exponential phase is dependent on the environmental conditions and the organism. In vitro, unrestricted (exponential) growth in chemostat cultures is amenable to quite sophisticated mathematical analysis (e.g., 35, 57, 93).

Bacterial physiological responses to environmental changes have mainly been studied using exponential phase cells. Where these changes are potentially lethal, the responses are referred to as stress responses. The genetic basis for the phenotypic changes elicited by environmental change has been studied extensively, initially by mutational and reporter analysis and at the proteomic level (10, 32, 40, 41, 115, 127). Latterly, genomic and subgenomic arrays have afforded an attractive approach to studying these adaptive responses at a global transcriptional level (119). Depending on the nature of the environmental change or stress, the changes in gene expression elicited may involve between tens and hundreds of different genes. Where the change is not stressful (as defined above) it appears that growth is substantially slowed down or arrested and resumes after the adaptation is complete. In some cases, notably where nutrient depletion precludes further growth, changes in the pattern of gene expression are not confined to a single shift but rather a sequential programme of change is entered into (54). Where this results in a defined morphological adaptive change, such as in sporulation, it is referred