THE SCOUT MODEL: A NEW VIEWPOINT ON MICROBIAL SURVIVAL STRATEGIES

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by

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Abstract:

It has been frequently observed that microorganisms from different sources exist in a non-growing state. We suggest that this state represents a population level strategy utilized by non-sporeforming bacteria to maximize survival under unfavorable conditions. Individual members of the population enter a state of reversible dormancy from which they exit in a seemingly random fashion due to noise in gene networks. We term these stochastically awakened cells scouts, and further hypothesize that scouts may produce signaling compounds that cue the remaining dormant population to initiate growth (Epstein 2009a). To test this model, we employed single cell incubations from a mixed marine sediment sample, a model organism for the pathogen *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, and *Escherichia coli*.

Single cells of environmental samples from the top oxic layer of the marine sediment incubated over extended times under static conditions showed initiation of growth well beyond typical growth times, ranging up to 18 months. Identification of isolates via sequencing of the 16S rRNA gene revealed that the majority of late growing isolates were typical marine species with known growth rates well below what was evidenced in our experiments. Furthermore, upon subculture the majority of the isolates (>60%) grew in under 1 month regardless of their initial time required to visualize growth. *Escherichia coli* cells treated in various ways (antibiotic challenge, exposure to environmental conditions, etc) resulted in a stark increase in the non-growing population relative to total cell counts. Isolation of single cells showed that individual cells were able to initiate growth at seemingly random intervals stretching up to 2 months. This
indicates that *E. coli* exhibits recovery in a manner consistent with the scout model. Further development of a model utilizing *E. coli* would be extremely useful for downstream studies into the mechanism of dormancy. Dormant *M. smegmatis* cells, incubated at various concentrations, exhibited visible growth at time points ranging from the typical 48-72 hours, up to over 2 months, in a linear fashion. An ethyl acetate extract of spent medium from actively growing mid-exponential phase *M. smegmatis* cultures applied to dormant cells resulted in an increase of cells exhibiting visible growth after 5 days of incubation. Methanol fractionation of the extract has allowed us to close in on the potential molecule causing this activity.

These experiments indicate that reversible dormancy consistent with the proposed scout model is present in bacteria from diverse sources, giving us insight into bacterial behavior, potentially leading to the cultivation of previously uncultivated species from the environment, as well as offering a different approach to developing treatments for recalcitrant infections.
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Dedication

This dissertation is dedicated to my children, Isabella and Gabriel Atencio-Earnshaw, who inspire me everyday.
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Cells challenged with ampicillin resume growth continuously after removal of the antibiotic.

Two independent long-term experiments show that dormant cells of *M. smegmatis* initiate growth at apparently random time points, with cumulative growth curve exhibiting no sign of leveling off even after 3 months of incubation.

Two independent long-term experiments show that dormant cells of *M. smegmatis* initiate growth at apparently random time points, with cumulative growth curve exhibiting no sign of leveling off even after 3 months of incubation.

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Section 1. Introduction and Background

The principal goal of this project is to provide support for the recently proposed scout model of microbial life cycle (Epstein 2009a, 2009b). I achieve this by checking the predictions of the model using both environmental and standard lab strains (Escherichia coli and Mycobacterium smegmatis). As I will argue below, the validation of scout model may have important implications in the understanding of how microbes survive challenges in the environment and in the human body. In this section, I will introduce the model, and discuss its implications. I begin by examining the phenomenon of dormancy in bacteria.

Section 1.1 Dormancy in Bacteria

Dormancy is a phenomenon that has been postulated in bacterial cells repeatedly, but exact definitions are lacking. While the presence of a non-growing subpopulation has been clearly shown for a number of bacterial species, the definition and subsequent proof of dormancy is more complicated. Here, I define dormancy as a non-growing state from which the cell can exit, and resume growth. Most important is the continued viability of the cell and its ability to emerge from this state, without which it is difficult to differentiate between a dormant and a dead or dying cell. There are a number of lines of evidence that point towards the existence of a dormant state in bacteria, making this reversible state extremely plausible.
Various studies of environmental samples have shown an exceptionally slow overall growth rate, with the mean generation times calculated to be on the order of thousands of years for deep sea sediments (Jørgensen et al. 2007; Jørgensen 2010) and up to 200 hours for bacteria from open sea water (Jannasch 1969; Riemann et al. 1987). Of course, the bacteria that are regularly isolated from environmental sources show a much faster growth rate. Indeed studies have estimated that up to 98% of microorganisms are in a non-growing state (Novitsky 1987). Accordingly, average growth rates would have to be significantly adjusted in order to account for such a large portion of non-growing cells.

Furthermore, it has been observed repeatedly that microscopically visible cells from the environment fail to grow on traditional media. This phenomenon, formally termed ‘The Great Plate Count Anomaly’ by Staley and Konopka in 1985, appears in the literature as far back as the late 19th century (Winterburg 1898). Winterburg and many others after him (Razumov 1932; Amann 1911) observed higher numbers, by several orders of magnitude, in direct microscopic counting versus plating. It is likely that some of these microbes fail to grow due to inadequate growth conditions. However, novel species continue to be recovered using unmodified, conventional techniques (for example: Kumar et al. 2007). The question then is, why do these newly discovered species grow on some but not other occasions, given that the cultivation approaches used were the same? An explanation could lie in the presence of dormant cells. Incidentally, if cells of those species were present only as dormant cells in previous experiments, we could expect that subsequent platings may result in the recovery of novel species without any
new techniques. An interesting implication is that, if the above is correct, then the
discovery of new species using conventional techniques is merely a matter of plating
enough cells to get a non-dormant representative.

Another hypothesis relevant to microbial dormancy is that of ‘Viable but Not Cultivable
Bacteria’ or VBNC state. This hypothesis asserts that cells exist in the environment in a
state in which they maintain viability (and in the case of pathogenic strains, virulence),
but are not detectable using traditional techniques (Xu et al. 1982; Oliver 2005). This
hypothesis specifically addresses many bacteria that are known to have the ability to
grow in the laboratory using basic, well-established techniques. Numerous studies
have lent support to this hypothesis, and the VBNC state has been invoked to explain
behavior in a plethora of both pathogenic and non-pathogenic bacterial strains. VBNC
studies have established viability in non-growing cells through the presence of intact
membranes (i.e BacLight Live/Dead assays) and using reverse transcriptase (RT)-PCR
and other molecular techniques (Oliver 2010).

Bacteria treated with antibiotics provide perhaps the most convincing evidence for the
existence of dormant subpopulations of bacteria. Indeed, dormancy is now the
accepted explanation for the survival of a small portion of cells treated with antibiotics,
known as persister cells (Lewis 2010; Balaban et al. 2004; Shah et al. 2006). Persister
cells are a subpopulation that survive a challenge by a high dose of antibiotics, and
grow immediately after removal of the antibiotic. These cells foster a new population
that shows the same susceptibility to antibiotics as the original culture, indicating that
genetic mutation or acquired resistance are not responsible for the lack of action of the antibiotic (Bigger 1944; Lewis 2010). As antibiotics target the processes of actively replicating cells, persister cells are able to evade the action of the antibiotic by existing in a non-replicating state (Lewis 2010). Visualization of individual cells through the use of a microfluidic device showed conclusively that at least some persisters were indeed in a non-growing state before the application of antibiotics (Balaban et al. 2004).

The existence of latent infections further evidences that subpopulations of dormant cells exist in pathogenic strains. Recalcitrant infections are well documented and can be explained by the existence of a subpopulation of cells that both evade the immune system of the host and are unaffected by treatment. One way for cells to do this is to exist in a state of dormancy. A particularly potent example is that of the pathogen *Mycobacterium tuberculosis*, the causative agent of tuberculosis. Tuberculosis patients can harbor bacteria for years without symptoms, only to show active infection long after initial exposure (Wayne et al. 1996; Chao et al. 2010). Indeed, molecular strain typing in patients with infections showed that the same strain could cause re-infection up to 33 years after initial exposure (Lillebaek et al. 2002).

Based on the above described observations, we can see that dormancy is likely prevalent in the bacterial world, and is a parsimonious explanation for a number of commonly observed phenomena. However, while the existence of dormancy is not disputed, there is no established explanation for how cells exit this state. Signals and cues from the environment are widely assumed to play a role, however this explanation
is not satisfactory to explain a number of observations. Persisters, for example, are
dormant cells that exhibit growth immediately after the removal of antibiotics in the
same medium in which they failed to grow only hours earlier (before addition of
antibiotics). To address these and other inconsistencies described below (Section 1.3),
we proposed the scout model (Epstein 2009a, 2009b).

Section 1.2 The Scout Model

We propose that cells in a population exist in at least two states or subpopulations
(Figure 1.1). When conditions are permissive, the population consists of a majority of
actively growing cells and a subpopulation of dormant cells (Step I). If conditions
change and become adverse, some cells will die off, but others will go into dormancy,
increasing the pool of non-growing cells (Step II). We postulate that these non-growing
cells will exit dormancy in random, stochastic pattern, and the resulting active (‘scout’)
cells are the key element of the model (Step III). If conditions remain adverse, the
awakened scout will soon die (Step IV). Individual cells from the non-growing
subpopulation will continue to randomly awake, such that a small subpopulation of
‘scouts’ will be continuously testing the environment, until one of them encounters
favorable conditions (Step V), and re-establishes the population (Step VI). This
proposed strategy would be useful in allowing populations to persist in environments of
constant changes and challenges, ensuring propagation even if the dormant cells lack
mechanisms of detecting conditions conductive to growth.
We further propose that growing scouts in at least some species may have the ability to induce the remaining dormant population to initiate growth. In this scenario, an awakened scout begins to grow and upon reaching a specific cell density accumulates enough of an e.g. signaling compound that induces dormant cells to growth (Fig. 1.1; step VI-VII). As a side note, there is of course a possibility that the signal may also come from a different species in a e.g. synergistic relationship. Examination of this possibility is outside the scope of this project.

Figure 1.1. Microbial strategies of survival in nature: dormancy under adverse conditions, growth under favorable conditions, with transition via scout cell and/or growth-promoting signaling compounds. Artwork by Stacie Bumgarner, Whitehead Institute for Biomedical Sciences Cambridge, MA, USA (Taken from ‘General Model of Microbial Uncultivability’ Epstein, 2009).

The novelty of this proposed model lies in the idea of random, stochastically awakening cells. Although this model remains hypothetical, it is supported by studies that point to phenotypic individuality among members of genetically identical populations (Avery 2008; Dubnau et al. 2006; Xie 2010). This variation, termed bistability, has been attributed to stochastic fluctuations within the cells. Research suggests that this strategy has evolved in bacteria as a way to protect a genetically homogenous
population from adverse conditions by increasing the number of ways in which such population can respond to the challenge. Indeed, phenotypically heterogeneous populations do show an increased fitness when compared to populations that are phenotypically homogenous (Avery 2008; Dubnau et al. 2006; Veening et al. 2008). Bistability is the result of noise in gene networks (Dubnau et al. 2006; Xie 2010). By analogy, we hypothesize that exit from dormancy proposed in the scout model is also ruled by noise in gene networks, and may represent a general microbial survival strategy. One of the principal goals of this study is to obtain empirical support for randomness in microbial awakening.

The second aspect of the model that I will address in this project is growth induction of dormant cells by growing kin cells. There is increasing evidence that such induction may be widespread in microbial world. For example, Nichols et al. 2008 obtained two variants of the same environmental isolate MSC33, one of which was unable to grow on traditional media, whereas the other one was cultivable. Interestingly, in co-culture the latter induced the former to grow. It appeared that short peptides at nanomolar concentrations were involved in such growth induction, suggesting these peptides had signaling function. Other studies have had similar success with the application of supernatant from actively growing cultures, and in some cases elucidated the nature of activity (D’Onofrio et al. 2010; Mizunoe et al. 1999; Oliver et al. 1995; Roth 1988, Shah et al. 2008).

Section 1.3 Implications of the Scout Model
The scout model explains a number of previously unresolved paradoxes.

As described above, microscopically visible cells from the environment routinely fail to grow on traditional media. These ‘unculturable’ cells actually account for the vast majority of species that can be detected through molecular studies (Rappé and Giovannoni 2003). The scout model predicts that some environmental cells that we fail to cultivate represent a pool of dormant cells. Isolation of at least some novel species then is a matter of logistics. We must plate enough cells in order to get a scout cell. Alternatively, scout cells must be allowed to resume growth through long-term incubations.

The model explains some of the more controversial aspect of the VBNC hypothesis. An issue that has plagued the VBNC research is the general inability to get a truly non-growing culture. Among cells in VBNC state, there always remains a small percentage of cultivable cells. Given their fast response to growth-permissive conditions, their re-growth was sufficient to explain what others saw as resuscitation of VBNC cells (Bogosian 2001; Nyström 2003). The scout model offers a different view. It considers VBNC cells as dormant, and the cultivable fraction as scouts VBNC cells randomly produce, which explains why the two are inseparable. The model suggests that the cultivable fraction, instead of hindering the efforts of VBNC researchers, might have been the very evidence of awakening they sought.
The scout model also explains one peculiar feature of the classic microbial growth curve, a feature that is difficult to explain otherwise. In a closed system, a clonal culture will reach a maximum density at stationary phase followed by a decline in the number of viable cells after nutrients have been exhausted, and toxic waste accumulated. The existence of the ever decreasing number of viable cells is explained by nutrients leaking from the ever increasing number of dead cells. Note that such explanation, while entirely plausible, requires the negative slope of the growth curve, indeed observed after stationary phase. However, when the number of viable cells reaches a certain (low) level, the decline disappears, and viable cells continue to exist at a more or less constant density. Remarkably, this situation continues for years (Finkel et al. 2000). With no decline observed, the former explanation for the existence of viable cells cannibalizing their dead no longer applies. A natural question is, how, under the circumstances, can a cell maintain its activity for years? And, if some cells possess this almost supernatural ability to survive while remaining active, why did all the other cells had to die? The scout model offers a simple explanation. It postulates that at least some cells previously considered dead are in fact dormant, and the viable cells observed are stochastically formed scouts. Therefore, the viable cells registered for years in a capped vial did not have to be active during the entire death phase, are transient members of the population, and as such do not have to be assigned any miraculous property, which removes the paradox.

On a closer examination, the existence of persister cells also appears somewhat paradoxical, and the nature of this is not unlike the above example of the growth curve.
Persisters have been shown to be non-growing before and during the antibiotic exposure (Balaban et al. 2004). If so, why do they start growing after antibiotic has been removed? The scout model again postulates that the pool of dormant cells is significantly larger than the number of persisters typically counted, and explains that the latter are cells that stochastically awoke between the removal of the antibiotic and plating. It follows that persisters as operationally defined today may be no more than manifestation of a larger number of survivors that remain unseen, and are typically ignored.

The scout model is consistent with spontaneous reactivation of latent infection. Any resumption of growth of the (dormant) pathogen may be contained by the host’s immune system most of the time. At times, however, perhaps due to other illness, stress, etc, a growing scout of the pathogenic population may be able to evade the immune system, establish a new population and cause active infection in the host. This may have important implications for disease control, because if the mechanism of scout awakening were known, it might be possible to design a strategy of waking up all dormant cells, which could then be effectively killed by available antibiotics.

To summarize, the implications of the scout model may be important in both environmental and health studies, and so testing this model is of substantial interest. In this project, I provide experimental evidence in support of the scout model by checking its two key predictions.
The first prediction is somewhat counterintuitive: dormant cells, if exposed to growth-permissive conditions, will not initiate growth more or less simultaneously, will exhibit no species-specific lag phase, and instead will resume growth at random intervals spread throughout the incubation period. I check this prediction using three microbial models: environmental cells (Section 2), *E. coli* (Section 3), and *M. smegmatis* (Section 4).

The second prediction is that dormant cells of (at least some) microbial species can be induced to grow if exposed to a growing culture of kin cells. I check this prediction using *M. smegmatis* as a model organism, and additionally provide insights into the nature of the growth inducing activity (Section 5).

### Section 1.4 Single Cell Method

This project presented an important methodological challenge. Traditional studies in microbiology are done using a large number of cells, usually on the order of millions. Averages are taken for the entire population and then calculated as if they informed of the actions of the individuals (Zengler 2009). This is inherently inaccurate as it is known that bacterial cultures, even when genetically identical, can be phenotypically heterogenous (Dubnau 2006). The study of dormant cells especially necessitates the ability to look more closely at individual members of the population. Dormant cells do not grow at the time of observation making them essentially invisible by standard techniques. By the time they do initiate growth, the culture or the plate will be overgrown by the active cells that were growing from the beginning of the experiment.
Thus, observations are required at the level of individual cells. In order to conduct such observations, I developed a technique whereby cells from the culture in question are separated from each other in individual compartments. By isolation of individual cells, I was able to observe the behavior of single cells without the limitation imposed by overgrowth by faster growing cells. This technique is broadly applicable to cells from different sources, and to cells from mixed or clonal cultures, and was the key element of my research strategy.
Section 2. Marine sediment microorganisms: Single cell approach shows that a pattern of recovery of environmental species is consistent with the scout model.

Section 2.1. Introduction

As described in Section 1, the vast majority of environmental cells are thought to be in a non-growing state (Jørgensen et al. 1997; Stevenson 1978), and in the experiments described here I used environmental samples as a source of dormant cells. The scout model predicts that non-growing cells are capable of growth, will initiate division, but will do so at random time intervals, and irrespective of environmental cues. Consequently, a long-term growth experiment will exhibit cells from the same species forming colonies at widely different time points. It also predicts that, even if the initiation of growth occurs after prolonged incubation, this is not necessarily a sign of slow growth, the division rate does not have to be especially lengthy, and at least for some species the latter will equal that of commonly observed, fast growing species. This prediction cannot be tested by using conventional cultivation approaches, such as plating on standard Petri dishes, because fast growing species will quickly overgrow the dish and mask or inhibit growth of any cell that did not initiate its division soon after plating. However, if cells from the inoculum are cultivated in isolation, such as in different mini-Petri dishes or wells of a microtiter plates, then their growth will not be impeded by that of their neighbors, and the time they initiate growth can be registered. Whether or not species isolated late in the experiment do or do not represent inherently slow growing strains can be easily checked by observing how quickly subcultures form visible growth.
In order to observe growth of individual cells over time, I developed methods of incubating single cells from environmental mixes in both solid and liquid media. The following section describes two experiments that confirm the prediction of the scout model that cells from the same environmental species will initiate growth at various times, and without any distinct lag phase. This was achieved in collaboration with Ekaterina Gavrish, Northeastern University, Lewis Lab. DNA analysis for microtiter plate experiments was conducted in the Lewis Lab.

Section 2.2. Materials and Methods

Section 2.2.1 Isolation in solid medium: Teflon Chip experiments

Collection/preparation of samples. Marine microorganisms were obtained from the marine intertidal sand flat in the Massachusetts Bay in Nahant, MA USA (42°26’N, 70°56’W). Sediment was collected from the uppermost (oxic) layer at low tide during early spring 2006. A 0.5 g subsample was mixed with 2 ml of sterile sea water, and bacterial cells were separated from the sediment by 5 cycles of 15-minute vortexing/sedimentation, each time in a new 2 ml portion of sterile seawater, resulting in 10 ml of cell suspension.

Cell enumeration. Following general recommendations by Hobbie et al. (1977), samples for microbial enumeration were prepared by serially diluting the above cell suspensions in sterile seawater and fixing cells with 4% formaldehyde (Sigma, St.
Cells were stained by mixing 100 µl of a cell dilution, 20 µl of DAPI (4',6-diamidino-2-phenylindole) stain, and 2 ml of DNA grade water. After 10 minutes of staining in the dark, cells were collected on a 0.2 µm pore-size polycarbonate filters (Millipore, Billerica, MA). Filters were washed twice with DNA grade water, and mounted on standard glass slides. Filters were viewed at 1000x using a Zeiss Aksioskop 50 compound microscope equipped for epifluorescence. A minimum of 16 fields and 250 cells were counted for each preparation.

*Development of Teflon Chip.* In order to incubate single cells, we built a device (‘Teflon Chip’) that allowed for the quick loading and containment of single cells in small volumes of agar. The Teflon Chip is simply a sheet of Teflon with multiple through holes made using a hand drill. Dipping this chip into a cell suspension in warm agar is a straightforward way to load each through hole with an amount of agar defined by the size of the hole, and a number of cells determined by the cell concentration in agar. The most useful concentration for these experiments was on average one cell per through hole. The hydrophobic nature of Teflon provided that excess of agar dripped off the surface of the chip, so that the cells trapped in agar plugs became separated from each other.

Early prototypes contained from 5 to 40 holes of various diameter (Appendix, Figure A. 1). The prototypes were tested to determine (i) the optimal diameter of the hole and thickness of the Teflon sheet for loading of the chip, (ii) the required distance between
holes to avoid cross contamination, and (iii) the conditions under which the agar plugs in
the holes could be kept the longest without drying.

A series of tests involving through holes of varying diameter in Teflon sheets of various
thicknesses revealed that a good combination for loading was a hole of a 1 mm in
diameter on Teflon sheet 1.6 mm thick. Conveniently, such sheets can be autoclaved
without losing their flatness. Prior to autoclaving, the chips were washed in 70%
ethanol. All prototype chips were incubated in standard, 105 mm in diameter Petri
dishes. To prevent drying, each Petri dish contained a small reservoir of sterile water
from which the chip was kept separate.

Cross contamination of wells was tested by dipping half of a prototype chip into a
culture of a (highly mobile) species of \textit{Pseudomonas}, and the other half into sterile agar.
Wells were observed for growth over a two week time period.

The working version of the chip, as suggested by preliminary experiments, was 7 cm by
17 cm and 1.6-mm thick, with 250 holes spaced 5 mm apart (Appendix, Figure A.2).
Each through hole of such a chip captured 4.3 µl of agar.

\textit{Long-term, Teflon Chip-based cultivation experiment.} This experiment utilized 20 Teflon
Chips described above, collectively containing 5,000 through holes. Environmental
cells were diluted with 1% warm Bacto Agar (Benton-Dickson, Franklin Lakes, NJ)
supplemented with 0.1\% (v/v) Lysogenic Broth (LB Benton-Dickson, Franklin Lakes, NJ)
such that each 4.3 µl aliquot (volume of each hole in Teflon Chip) contained on average a single cell. Each Teflon Chip was dipped into the warm agar and slowly removed at an angle to fill the holes with agar plugs and allow any excess of agar to drip from the surface. After a short drying time, the chips were placed into glass vessels serving as humidifying chambers. A high humidity environment within the chambers was achieved by filling a small reservoir, separate from the rest of the chamber, with water, and placing a tight fitting cover on the chamber. The chips were incubated above the water reservoir in an autoclaved tray. Two chips filled with sterile warm agar served as negative controls, and were co-incubated with the chips containing cells.

**Incubation conditions, observations and removal of plugs.** Chips were incubated in humidifying chambers at room temperature for 4 months. They were removed and observed for growth, at 100x, at time points of 2 days, 5 days, 15 days, 3 weeks, 4 weeks, 6 weeks, 2 months, 3 months, and 4 months. Hereafter, growth observed in the agar plugs is referred to as *initial growth* (as opposed to *secondary growth* of subcultures, see below). Agar plugs exhibiting growth were removed using sterile needles and placed into 500 µl of autoclaved seawater containing 0.1% (v/v) LB. The plugs were syringe-disrupted, subjected to repeated vortexing, and divided into three parts in order to (i) create glycerol stock, (ii) subculture, and (iii) conduct downstream DNA analyses.
Glycerol stock library. Four 50 µl aliquots of disrupted material from each plug were separately added to 450 µl each of autoclaved 20% glycerol. Glycerol stocks were stored at -80°C.

Subculture. For each individual isolate, a single 500 µl glycerol stock was thawed on ice. A 100 µl subsample was taken from each stock and added to 500 µl of autoclaved sea water supplemented with 0.1% (v/v) LB, utilizing 24-well plates. Each plate had three wells that contained 500 µl of sterile medium as a negative control, and three wells with 500 µl of sterile medium plus Escherichia coli cells as a positive control. Plates were sealed with Parafilm and incubated at room temperature in humidifying chambers. Growth was observed over a one week period.

DNA analyses. Isolates recovered in the experiment were identified via sequencing of their 16s rRNA gene. For each time point, and to minimize costs, materials from the agar plugs were pooled, and the 16s rRNA genes were PCR-amplified using universal eubacterial primers 27F (5’-AGA GTT TGA TCC TGG CTC AG-3’) and 1492R (5’-GGT TAC CTT GTT ACG ACT T-3’) and Platinum PCR SuperMix (Invitrogen, Carlsbad, CA). Amplicons were cloned using a TA cloning kit with TOP10 cells (Invitrogen, Carlsbad, CA). Ninety-six white colonies per library were selected and PCR-amplified using universal M13F (5’-GTT TTC CCA GTC ACG ACG TTG TA-3’) and M13R (5’-CAG CAA ACA GCT ATG ACC-3’) primers. Sequencing was done commercially by SeqWright, Inc. (Houston, TX). Sequences were edited using the BioEdit program (Tippmann 2004), and pairwise sequence alignments made using ClustalW (Thompson et al. 1994).
at default settings. The percent sequence similarities were calculated, and the sequences were clustered into OTUs using the mean unweighted-pair group method using average linkages. OTUs formed on the basis of >97% gene sequence identity were considered to represent species (Stackebrandt and Goebel 1994). OTU identity was established by comparing representative sequences to the GenBank database using the BLAST search.

**Section 2.2.2 Isolation in liquid medium: microtiter plate experiments**

*Collection/preparation of samples/cell enumeration.* Marine microorganisms were obtained in the fall of 2007, and cells were separated from the sediments and counted as described above.

*Preliminary experiments to determine optimal nutrient concentration and temperature conditions during plating.* Sediment samples were plated on Petri dishes containing 1.5% bacto agar supplemented with 0.01, 0.1, 0.5 or 2.5% (v/v) LB. Incubation in 0.1% (v/v) LB resulted in higher total colony forming units count (cfu) and higher morphological diversity of colonies. Since the eventual administration of cells into microtiter plates was going to take about three hours (see *Loading of the Microtiter Plates*), and therefore some cells would have to be stored for up to three hours before the experiment commenced, I determined temperature conditions that would prevent microbial growth during this three hour period. Aliquots of cell suspensions were incubated at room temperature (~25°C), and also at 0, 4, 8, 12, 14, and 16°C, and from
each suspension subsamples were withdrawn at times ranging for time zero to three hours. I determined that when cell suspensions were kept at 8, 12, or 16°C, colony counts at time zero and three hours were statistically the same, whereas incubations at lower and higher temperatures resulted in, respectively, reduced and elevated colony counts. I chose 12°C as an appropriate temperature to store cells while administering them into microtiter plates.

**Loading of the microtiter plates.** Environmental cells were mixed with autoclaved natural sea water supplemented with 0.1% (v/v) LB to an approximate density of one cell per 50 µl. A 12-channel micropipetter was used to distribute 50 µl aliquots of thus obtained cell suspension over 31 384-well microtiter plates. In each plate, 360 wells received the above aliquots, whereas the remaining 24 wells were filled with sterile medium as a negative control. The entire procedure took under three hours, during which the cell suspension sampled was kept at 12°C. The loaded plates were sealed with Parafilm and incubated at 12°C, which represented the ambient temperature at time of collection.

**Observation of growth.** Plates were inspected at monthly intervals for growth visible to a naked eye, and all instances of growth were confirmed under a dissecting microscope (Zeiss Stemi 2000). Wells exhibiting growth were marked, and the time of growth appearance recorded.

**Removal of colonies.** To minimize the risk of contamination, and assuming that a single lifting of the plate’s cover might lead to contamination, the plates were opened for
growth removal only once, and then sacrificed (with the exception of the last 7 plates, see below). The plates were divided into 3 subsets. The first subset (12 plates) was opened for growth removal after 5 months, the second (12 plates) after 6 months, and the final subset (7 plates) after 13 months incubation. As we reached, with the last subset, our target incubation period of 1 year, and the risk of contamination was no longer crucial, I resealed this last group of plates and incubated them for an additional 6 months in the hope that control wells would not show contamination. No contamination was observed, allowing for an additional sampling at the 18 months time point. The experiment was then terminated due to excessive drying of the nutrient medium.

Upon opening of the plates, material in wells showing microbial growth was removed using a micropipette. Repeated pipetting and disruption of any visible material at the bottom or edge of the well ensured that the entire contents of the well were removed. For each well, the removed material was placed into a sterile 1.5 ml tube and vortexed. This material was then divided for the following purposes: (i) creation of a glycerol stock (ii) subculture and (iii) freezing at -80°C for downstream DNA analysis. Material from select wells was also used for: (i) enumeration of cells in the well for subsequent single cell incubation and (ii) microscopic verification of the presence of cells.

Verification of growth. A portion of wells scored positive exhibited very minimal growth, which could in principle be confused with accumulation of precipitate in the medium. To reject this and confirm microbial growth, select wells were observed for the presence of
cells using Zeiss Aksioskop 50 compound microscope at 1000x magnification and employing Differential Interference Contrast (DIC).

**DNA analyses.** Isolates recovered in the experiment were identified via sequencing of their 16s rRNA gene. PCR-aided amplification was performed on each isolate using universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'), or semi-nested PCR-aided amplification using 27F and 1492R, followed by PCR-aided amplification using 27F and 907R (5'-CCG TCA ATT CCT TTA AGT TT-3'). In some case, PCR failed under above conditions, and then an alternative forward primer 357F (5'-CCTACG CGA GGC AGC AG-3') was used. Sequences were obtained commercially. Sequences were entered into a FASTA file and imported into the ARB database (Ludwig et al. 2004), and species were identified as the smallest clusters of sequences on the phylogenetic trees, roughly corresponding to clades based on 97% sequence similarity cut off value. Representative sequences from each clade were compared to the GenBank database using the BLAST search.

**Subculture of samples.** The portion of each well’s growth designated for subculturing was, upon removal from the well, immediately inoculated into a 1.5-ml vial pre-filled with fresh medium, incubated for 9 months, and periodically observed for growth. The time point at which visible growth first appeared was recorded for each isolate.

**Heterogeneity experiments.** In order to establish whether or not subcultured cells of a given isolate were heterogenous or would initiate growth simultaneously, I established
‘heterogeneity experiments’ as follows. I randomly chose 20 wells showing microbial growth from the second subset of microtiter plates, and enumerated cells in each via DAPI staining (as described above). Cells were then mixed with autoclaved natural sea water supplemented with 0.1% (v/v) LB to an approximate density of one cell per 50 µl. A 12-channel micropipetter was used to add 50 µl aliquots of thus obtained cell suspension to 360 wells in a 384-well microtiter plate for each sample. The top row (24 wells) was filled with sterile medium as a negative control. The plates were sealed with parafilm and incubated at 12°C, and observed for growth in the manner described above at 1 day, 6 days, 11 days, 18 days, 1 month, 2 months, 2.5 months, 3 months, 4 months, 5 months and 6 months.

Section 2.3. Results

Section 2.3.1 Isolation in solid medium: Teflon Chip experiments

Section 2.3.1.1 Preliminary experiments

Cross contamination of wells does not occur. Teflon Chips loaded with sterile agar and a mobile cells of *Pseudomonas* showed no contamination of the (initially) cell-free wells after an incubation period of 2 weeks.

Section 2.3.1.2 Long-term growth experiments
Cell enumeration. The number of cells removed from the marine sediment sample for the Teflon Chip experiments corresponded to $4.62 \times 10^7$ cells per gram of sediment.

Growth recovery of environmental cells. The 20 Teflon Chips used in this experiment contained 4,704 agar plugs (agar presence in each through hole was confirmed by visual inspection), and thus 4,704 cells. The first colonies appeared after 48 hours of incubation, and new growths were observed throughout the four months of incubation (Figure 2.1), totaling 175 colonies (3.72% of the cells inoculated). The majority of the growths were in the form of single colonies, although multiple colonies were observed in a few cases.

![Figure 2.1. Increase in the number of colony forming units observed in agar plugs over four months of incubation of Teflon Chips (percent of total agar plugs inoculated).](image)

Species identification reveals same species appearing at different time points. A total of 13 species were recovered. A number of typically fast growing species appeared at late
time points, such as two *Bacillus* sp. that did not appear as visible growth until the 120th day of incubation. Two *Pseudomonas* species appeared at multiple time points, one of which, *Pseudomonas argentensis* strain CH101 appeared at all 8 time points (Figure 2.2).

<table>
<thead>
<tr>
<th>Teflon Plates</th>
<th>Bacillus</th>
<th>Planococcus</th>
<th>Pseudomonas</th>
<th>Pseudomonas</th>
<th>Pseudomonas</th>
<th>Pseudomonas</th>
<th>Pseudomonas</th>
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<tr>
<td></td>
<td>Bacillus sp.</td>
<td><em>Planococcus</em> sp.</td>
<td><em>Pseudomonas</em> argentensis strain CH01</td>
<td><em>Pseudomonas</em> argentensis strain CH01</td>
<td><em>Pseudomonas</em> argentensis strain CH01</td>
<td><em>Pseudomonas</em> argentensis strain CH01</td>
<td><em>Pseudomonas</em> argentensis strain CH01</td>
<td><em>Pseudomonas</em> argentensis strain CH01</td>
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<tr>
<td>Teflon Plates</td>
<td>Day 2</td>
<td>Day 5</td>
<td>Day 11</td>
<td>Day 21</td>
<td>Day 28</td>
<td>Day 42</td>
<td>Day 90</td>
<td>Day 120</td>
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<tr>
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<td>Vibrio sp. V514</td>
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</tbody>
</table>

Figure 2.2 Recovery of species over time from Teflon Chip agar plugs. Top Row (months) represent time at which agar plug was observed to display growth.

**Microbial novelty.** While some species cultivated in this experiment were close relatives of previously isolated species, other were novel. For example, a very novel strain appeared at Day 5, with the closest cultured relative *Bacillus* strain RS2, sharing with it as little as 92% of 16S rRNA gene sequence identity.

**Subculture reveals majority of isolates are capable of fast growth.** The majority (60%) of the isolates subcultured exhibited growth within 24 hours; an additional 28% grew within
the next 24 hours. This speedy growth was in sharp contrast to the length of the initial incubation period (Figure 2.3).

Figure 2.3. Upon subculturing, a majority of isolates are able to grow after only 48 hours of incubation, irrespective of the time their required to grow in the initial Teflon Chip experiment. Initial incubation represents agar plugs displaying growth on Teflon Chips; Secondary incubation represents subculture of disrupted agar plugs incubated in liquid medium.

Section 2.3.2. Isolation in liquid medium: microtiter plate experiments.

Section 2.3.2.1 Preliminary experiments

Determination of optimal storage temperature during plating. Enumeration of cells at various temperatures over a 3 hour time period showed that storage between 8-16°C resulted in statistically stable counts (Figure 2.4).
Figure 2.4. Change in total cell count in a marine sediment sample over time at various temperatures. Samples were collected and enumerated, then stored at a specific temperature for 2 or 3 hours then enumerated.

Storage of the sample at 25°C resulted in an increase in cfu upon plating after 2 and 3 hours. Storage of the sample at 4°C and 0°C resulted in a decrease in the number of cfu upon plating after 3 hours. Storage at 16°C resulted in a stable cfu count upon plating at 2 and 3 hours (see figure 2.5).

Figure 2.5. Percent of microscopically visible cells exhibiting growth after 24 hour incubation as colony forming units over time at various temperatures.
Determination of optimal nutrient concentration for total recovery and diversity of morphologies. More colonies grew at lower concentrations of LB (0.1% and 0.5%) than at higher LB concentrations, but the difference was not statistically significant (Figure 2.6). The diversity of colony morphologies was the greatest in Petri dishes supplemented with 0.1% LB agar.

![Figure 2.6 Percent of microscopically visible cells exhibiting growth as colony forming units over time on agar supplemented with 0.1, 0.5, or 2.5% LB.](image)

Section 2.3.2.2 Long-term growth experiment

Cell enumeration. The number of cells removed from the marine sediment sample for the microtiter plate experiments corresponded to $1.49 \times 10^7$ cells per gram of sediment.

Overall microbial recovery. All 3 subsets of microtiter plates showed new instances of growth at all time points, with the total recovery standing at 4.0% after a 5 month incubation, 3.3% after a 6 month incubation, and 7.1% after a 18 month incubation (Figure 2.7).
Verification of microbial growth. In those instances when it was not clear if the material scored as growth was indeed microbial biomass, direct microscopy verified presence of cells. This is illustrated in Figure 2.8. Additional examples of photographed growth are in Appendix, Figures A.3-5.
Figure 2.8. Direct microscopy reveals that cases of ambiguous growth were, in fact, microbial biomass, as illustrated by photos of two isolates obtained after four and eight months of incubation.
**DNA analyses.** In total, I recovered 310 isolates comprising 86 different species, with 37 species recovered at multiple time points. Of the remaining 49 species, 45 species were represented by a single isolate, and five by multiple isolates. The time span over which selected species were recovered is shown in Figure 2.9. A full list of species recovered is provided in Appendix, Figure A.6.

![Figure 2.9. Examples of species whose growth was registered at multiple time points. Top Rows indicate the time at which growth was observed in the well. Blue boxes with numbers indicate that growth of the species was observed at that time point. Numbers in the blue boxes indicate the number of wells in which growth was observed at each time point.](image)

**Microbial novelty.** Of the 86 species recovered, 11 were novel, with various degree of phylogenetic novelty. Examples include substantial novelty of isolates sharing as little
as 84% 16S rRNA gene sequence identity with *Balneola* sp. YCSA29; 85% identity with *Sphingobacteriales bacterium* KIP; 94% identity with *Gaetbulibacter* sp NH57N; 95% identity with *Ulvibacter litoralis* strain KMM 3912, *Gilvibacter sedimini*, and *Winogradskyella* sp. 69Xa1-Y1; and 96% identity with *Eudora* sp. MOLA 359, *Lewinella agarilytica*, Arctic sea ice associated bacterium ARK, *Roseobacter* sp. UAxFpsK-5, and Alpha proteobacterium MBIC3952.

*Individual cells of isolates exhibit different degrees of heterogeneity within populations with respect to initiation of growth.* Incubation in the single cell format showed heterogeneous behavior of cells within most species. Some species showed individual cells exhibiting growth at dramatically different time points spanning the 5-month incubation period, while other species showed a majority of their cells initiating growth early in the incubation, followed by only a few additional instances of visible growth later on. In some species, only few cells showed growth at all (Figure 2.10). A full list of species tested in this single cell format, and the time points at which visible growth was noted is provided in Appendix, Figure A.7.
Figure 2.10. Increase in the number of wells showing growth for individual species over 90 days of incubation. Individual species were diluted to approximately a single cell per well in microtiter plates and observed over a 90 day period for visible growth in the wells.

Subculturing reveals that majority of isolates are capable of fast growth. Upon subculturing, the majority of isolates (60%) exhibited growth in the first month. Compare this to a mere 0.6%- the proportion of all isolates that exhibited growth within the first month of the initial incubation (Figure 2.11).
Figure 2.11. Comparison of the initial and secondary growth rates. Initial incubation represents wells in the microtiter plates that exhibited visible growth; Secondary incubation represents subculture of material from the wells.

The time an isolate required to exhibit visible growth during initial incubation was often different from that during subculturing of the isolate. Representatives of the same species showed variable secondary growth rates once subculture. For example, four isolates identified as *Winogradskyella echinorum* strain KMM 6211 took 1, 2 and 21 days to re-grow, respectively (Figure 2.12).
Figure 2.12. Comparison of initial versus secondary growth rate for select isolates.

Section 2.4. Discussion.

The two long-term, single cell based growth experiments were designed to check the principal prediction of the scout model: provided environmental samples contained a significant number of dormant cells, at least some species would be recovered multiple times, and at time points scattered over the length of incubation. Both solid and liquid media based experiments confirmed this prediction.

In Teflon plates, we observed *Pseudomonas argentinae* strain CH01 appearing at all time points, and *Pseudomonas* sp. E4-1 at 4 time points (Figure 2.2). In microtiter plates, 34 species appeared at multiple time points. For example, *Plantibacter cousiniae* and *Psychroserpens* sp. A622 both appeared at 8 time points ranging from 2 to 13 months and 5 to 13 months respectively. Collectively this suggests that cells in
each population were in a non-growing state at the beginning of the experiments, remained in this state for variable times, and initiated growth in an apparently random fashion.

A possibility remained that cells of those species that formed colonies at widely different time points represented strains inherently different in their growth rates. We checked this possibility by subculturing colonies of species recovered multiple times, and observed that their secondary growth rates were similar regardless of the time they required to form visible growth in the initial long-term experiment. For example, one isolate of *Plantibacter cousiniae* took 8 months to form visible growth in the initial incubation, but showed visible growth upon subculture in 24 hours, and an isolate of *Winogradskyella echinorum* strain KMM 6211 took 3 months to form visible growth in the initial experiment, but only 48 hours upon subculture. Therefore, the slow growing nature of organisms, suggested by the late appearance in the long-term experiments, may be only apparent, and at least some ‘slow growers’ are as capable of fast expansion as well known ‘weed’ species.

Collectively, this strongly indicates that growth behavior of a number of species recovered in the long-term experiments is consistent with the scout model of microbial life cycle. This has several implications.

First, at least in some cases, the distinction between slow growers and fast growers no longer appears certain. Indeed, the only reason I learned that *Pseudomonas*
argentinesis strain CH01 from the Teflon Chip experiment can appear as both is that
this strain was represented in the initial inoculum by multiple cells, an obvious
prerequisite for observing the strain multiple times. If this strain was represented by a
single cell, the colony it formed would have an equal probability of appearing early or
late in the experiment. In the first case, a standard interpretation would be that the
strain is a fast grower, in the second that it is a slow grower, while in fact it is neither.

Second, if a species is rare, represented only by a few cells in an inoculum, and these
cells are dormant, it may be exceedingly difficult to isolate such species using
conventional approaches. Its colony(-ies) may be formed at any time, including late in
the growth experiment. By that time, fast growing species may well overtake Petri dish,
masking the arrival of the new colony. Note that this rare species is still detectable in
the inoculum by molecular techniques. Its presence in clone libraries, and absence in
culture collection can easily be interpreted as ‘uncultivability’, at least on the nutrient
medium employed, which may be an erroneous conclusion. The only plausible
possibility to ever detecting a colony of such species would be if by chance its dormant
cells formed a scout right at the time of plating. This means that species discovery
should at times appear random, cultivation technique independent. In fact, this is
precisely what the collective experiences of cultivation microbiologists suggest. For
example, the phylum Verrucomicrobia was considered ‘uncultivable’ for many years,
until a few strains were isolated by Janssen et al. 1997, curiously, by the very same
techniques that had been quite unsuccessful prior to that work.
Third, my observations suggest one way to change such random discovery into a more controlled process. If the above logic holds, then the Petri dish may simply be a suboptimal tool. Isolation of a relatively rare organism, given predominance of dormancy in environmental sample and random exit from it, dictates the need to separate cells from each other in order to give every population a chance to form a scout. Single-cell based cultivation, analogous to the one used here, appears to be a good tool to achieve just that. In fact, some of the species I isolated in this project proved very novel, for example one isolate showed only a 84% similarity to its closest relative in GenBank, *Balneola sp.* YCSA29, another showed only a 85% similarity to its closest relative in GenBank, *Sphingobacteriales bacterium* KIP.

In conclusion, evidence presented here is consistent with predictions of the scout model of the microbial life cycle. This is important because validation of the model has implications for environmental microbiology, from shedding light on the nature (and reality) of fast growers vs slow growers, to interpreting the nature of uncultivability of some environmental species, to informing future cultivation efforts.
Section 3. Dormant cells of *Escherichia coli* K12 3W110 resume growth at substantially different time points.

Section 3.1 Introduction

The results from the marine sediment sample experiments showed that the scout model may be applicable to environmental bacteria. However, environmental species are mostly novel, and resolving mechanisms of their growth control presents practical difficulties not found in standard laboratory model species. For example, well developed tools for studying molecular underpinnings of behavior in *Escherichia coli*, such as knock-out and over-expression mutant libraries, make this species very attractive as a model organism. It was therefore important to establish whether *E. coli*, similarly to environmental microorganisms, exhibited growth behavior consistent with the scout model. In particular, I was interested in finding out how to put cells of *E. coli* into reversible dormancy, and observing the pattern of awakening of such cells.

I explored several ways of inducing dormancy in *E. coli*, and/or separating its growing and non-growing cells. VBNC research contains numerous examples of induction of dormancy, such as by nutrient starvation, changing osmotic pressure, exposure to visible light, and incubation in environmental conditions (i.e. seawater) (Kjelleberg et al. 1987; Mizunoe et al. 1999; Munro et al. 1989; Na et al. 2006; Oliver 2005). I attempted to reproduce the reported ways to induce dormancy in *E. coli*. Also, it seemed logical to explore whether cells in late stationary phase were enriched for dormant cells. In parallel, I experimented with density centrifugation as a method to separate *E. coli* cells in different physiological states, in hope that such separation would enrich for dormant
cells. This is based on a study by Makinoshima et al. 2002, which reported 15 discrete bands formed in density gradients to which stationary phase cultures of *E. coli* were applied, and also other studies indicating that non-growing and growing subpopulations are possible to separate (Arana et al. 2008; Cuny et al. 2003; Desnues et al. 2005). Below I describe these attempts to induce/obtain/enrich for dormant cells of *E. coli*, as well as an alternative way of getting such cells via killing growing cells by antibiotics, which ultimately became my method of choice.

**Section 3.2 Materials and Methods**

**Section 3.2.1. Strains, growth conditions, cell enumeration and cultivation**

*Strains, medium and growing conditions.* *Escherichia coli* K12 W3110 was kindly provided by the Lewis Lab (Northeastern University). Cultures were grown in autoclaved LB for either 3-4 hours (exponential phase) or 16-18 hours (stationary phase) at 37°C, with shaking at 250 rpm.

*Cell enumeration.* Following general recommendations by Hobbie et al. (1977), samples for cell enumeration were fixed with 4% formaldehyde (Sigma, St. Louis, MO), and stained by mixing 100 µl of a cell suspension, 20 µl of DAPI (4',6-diamidino-2-phenylindole) stain, and 2 ml of DNA grade water. After 10 minutes of staining in the dark, cells were collected on a 0.2 µm pore-size polycarbonate filter (Millipore, Billerica, MA). Filters were washed twice with DNA grade water, and mounted on standard glass
slides. Filters were viewed at 1000x using a Zeiss Aksioskop 50 compound microscope equipped for epifluorescence. A minimum of 16 fields and 250 cells were counted for each preparation.

**Loading of the microtiter plates.** Cells were mixed with sterile water supplemented with LB or LB agar to an approximate density of one, five, ten or twenty cells per 50 µl. A 12-channel micropipetter was used to distribute 50 µl aliquots of thus obtained cell suspension over 384-well microtiter plates. In each plate, 360 wells received the above aliquots, whereas the remaining 24 wells were filled with sterile medium as a negative control. The loaded plates were sealed with Parafilm and incubated at 37°C in humidifying chambers (described in Section 2). Wells exhibiting growth were marked, and the time of growth appearance recorded.

**Cultivable cell count.** Numbers of cells capable of growth within 24-48 hours was determined by plating onto Petri dishes with 1.5% bacto agar supplemented with LB. Number of cultivable cells was determined by counting colonies, and reported as cfu.

**Viable cell count.** Viable cell counts were made using Live/Dead BacLight™ Bacterial Viability Kit (Invitrogen, Carlsbad, CA). Samples for viable cell counts were prepared by serially diluting cell suspensions in sterile water and fixed with 4% formaldehyde (Sigma, St. Louis, MO). Stain was prepared following the protocol provided by the manufacturer. A 2x stock solution was prepared by dissolving the contents of the Component A (SYTO 9) and Component B (propidium iodide) pipets in 5 ml of sterile
water. Equal amounts of stock solution and bacterial suspension were combined and allowed to incubate in the dark for 15 minutes. Cells were collected on a 0.2 µm pore-size polycarbonate filter (Millipore, Billerica, MA). Filters were mounted on standard glass slides, and viewed at 1000x using a Zeiss Aksioskop 50 compound microscope equipped for epifluorescence. A minimum of 16 fields and 250 cells were counted for each preparation.

**Section 3.2.2 Induction of dormancy by stress**

**Section 3.2.2.1 Exposure to environmental stress conditions.**

*Nutrient starvation and osmotic pressure.* E. coli cells were grown in LB either to exponential phase (4 hours) or stationary phase (16-18 hours). Cells were diluted into sterile deionized water or sterile sea water to an approximate density of $10^7$ cells per ml and incubated at $4^\circ$C, room temperature ($\sim 25^\circ$C), or $37^\circ$C, with shaking at 250 rpm. Sampling was done weekly. Total cell count, cultivable cell count, and viable cell count were determined as described above.

*Starvation, osmotic pressure and visible light exposure.* Stationary phase cultures were diluted into either 10x PBS, 1x PBS, or sterile filtered sea water to an approximate density of $10^7$ cells per ml and incubated at room temperature ($\sim 25^\circ$C), with shaking at 250 rpm, and 10 cm away from a 26 watt bulb giving an output of 1450 lumens. Sampling was done at 24, 48 hours and weekly thereafter. Total count and
cultivable cell count were determined as described above. Microtiter plates were loaded with single and/or 10 cells per well, incubated, and observed for growth as described above at 24-48 hours and weekly thereafter.

Diffusion chamber incubation. Diffusion chambers were constructed as described in Kaeberlein et al. (2002). Stationary phase cultures were diluted into sterile filtered seawater to an approximate density of $10^7$ cells per ml, and 2.5 ml of cell suspension was added to the diffusion chamber. Diffusion chambers were incubated in a clear plastic container filled with unfiltered seawater collected from Canoe Beach, Nahant, MA. Chambers were removed from the container at 30 minutes, 1, 2, 4, 6 and 24 hour intervals or at 24 hours, 48 hours, and weekly intervals thereafter. The top membrane was peeled back using sterile tweezers, and the contents removed using a sterile syringe. Total count and cultivable cell count were determined as described above. Based on these counts, cells were distributed over 384-well microtiter plates aiming at administering either a single or 10 cells per well. The plates were incubated and observed for growth as described above at 24-48 hours and weekly thereafter.

Section 3.2.2.2 Starvation in late stationary/death phases

Late stationary phase experiments. A 10 µl loop was taken from an overnight culture and added to 20-40 ml of fresh medium in a 50 ml tube. Tubes were sealed and incubated at 37°C, with shaking at 250 rpm, for a minimum of 3 months. Total count and cultivable cell count were determined as described above. Microtiter plates were
loaded with single and 10 cell per well dilutions, incubated and observed for growth as described above at 24-48 hours and weekly thereafter.

*Incubation under anaerobic conditions.* Sealed, never opened cultures in late stationary/death phase (at least 5 months old) were used as a source of cells. Subsamples were removed, placed into a vinyl anaerobic airlock chamber (Coy Laboratory Products, Grass Lakes, MI) serially diluted, and cells were enumerated via DAPI staining as described above. Diluted samples were added to de-oxygenated LB, and diluted appropriately for microtiter plate incubations in single and 10 cells per well format. Sealed plates were placed in three anaerobic jars with anaero-gas packs (Mitsubishi Gas Chemical Company, New York, NY), which were removed from the anaerobic chamber, incubated at 37°C, and opened weekly to observe growth. Opening a box allowed oxygen to diffuse into microtiter plates, effectively transforming anaerobic cultivation into microaerophilic, with the amount of oxygen likely increasing over time. To reconstruct the time-dependent growth under anaerobic conditions, I opened a new anaerobic jar for each time point, but after counting, the plates were not discarded, and I kept scoring growth even as microaerophilic conditions were building up.

*Density separation.* Density separation was performed on cultures in late stationary/death phase. Cultures were spun in a Sorvall ST-H750 rotor at 4,000 rpm (3,313 g) in a Sorvall Super T21 Centrifuge (Thermo Scientific, Franklin, MA) for 10 minutes at 4°C to
concentrate the cells. Supernatant was discarded, and the cell pellet was reconstituted in 5 ml of fresh medium to concentrate cells.

Density gradients were prepared by mixing Percoll Plus (GE Healthcare Biosciences, Pittsburgh, PA) with 1x phosphate buffered saline (Sigma, St. Louis, MO) at concentrations of 20, 40, 50, 60, 70, 80, 90 and 100% Percoll, to a total volume of 9.5 ml in 10 ml Oak Ridge centrifuge test tubes designed to withstand 50,000 g (Nalge Nunc, Rochester, NY). Tubes were spun at 15,555 rpm (30,000 g), using a Sorvall SL-50T rotor with inserts for the smaller tubes, for 30 minutes at 4°C, thus creating a density gradient. Tubes were carefully removed to prevent disruption of the gradient, and 0.5 ml of the concentrated cell suspension was gently added. Tubes were spun in a Sorvall ST-H70 rotor at 4,300 rpm (3,829 g) for 10 minutes at 4°C. Tubes were carefully removed and bands noted. Bands were removed by inserting a sterile syringe into the tube and removing material from the top band, followed by subsequent lower bands.

**Section 3.2.3 Antibiotic treatment**

*Antibiotic stocks and dilutions.* Antibiotic stocks were prepared by dissolving ampicillin sodium salts, ofloxacin powder, or gentamicin sulfate (Sigma, St Louis, MO) into sterile water. The stocks were stored in 10 ml tubes at 4°C in dark conditions.
Minimum inhibitory concentration (MIC) of gentamicin. Minimum inhibitory concentration (MIC) for gentamicin was determined by following general guidelines from Wiegand et al. (2008). Gentamicin was added at concentrations ranging from 0.5 µg/ml up to 100 µg/ml to LB inoculated with *E. coli* in a 96 well microtiter plate. Plates were sealed and incubated at 37°C for 24 hours. Turbidity in the wells visible to the naked eye was scored as positive for growth. MIC was determined to be 0.5 µg/ml.

Antibiotic treatment. Cultures were grown to either mid-exponential phase or stationary phase. In selected experiments, we confirmed that cells were in these phases by counting cells using epifluorescence, as described above. Antibiotics were added to cultures at 50 µg/ml or 5 µg/ml for gentamicin, 500 µg/ml for ofloxacin or 100 µg/ml for ampicillin. Concentrations were determined based on the MIC and/or as advised by Dr. Iris Keren based on her experiences working with persister cells. Mid-exponential phase cultures were incubated for 3 hours in the presence of the antibiotic at 37°C, with shaking at 250 rpm. Stationary phase cultures were incubated for 6 hours in the presence of the antibiotic at 37°C, with shaking at 250 rpm (Keren et al. 2004a, 2004b). Antibiotics were removed by centrifugation in a Sorvall ST-H750 4,000 rpm (3,313 g) for 10 minutes at 4°C after which the supernatant was discarded and the cell pellet was resuspended in sterile LB. Centrifugation and resuspension was repeated twice for all samples. Resuspended cells were enumerated and placed into microtiter plates as described above using both liquid and solid media. Plates were sealed and incubated in humidifying chambers for 2 to 8 weeks, and growth was scored visually after 24-48 hours and weekly thereafter.
Section 3.3 Results

Section 3.3.1 Induction of dormancy by stress

Section 3.3.1.1 Exposure to environmental stress conditions.

Nutrient starvation with and without osmotic pressure results in stable cultivable cell, total cell and viable cell count over long-term incubation. Over a two week period *E. coli* cells incubated in autoclaved deionized water did not show a significant drop in the viable cell count as was reported by other studies (Oliver 2005). The cultivable cell count, total cell count, and viable cell count all showed no significant change over the incubation period (Figure 3.1). Replicates of this experiment confirmed my inability to replicate results reported in the literature (data not shown).
Incubation in artificial sea water without the addition of nutrients and autoclaved and/or sterile filtered natural sea water also did not show a drop in cultivable cells after incubations ranging from 1 to 4 weeks (data not shown).

Light exposure combined with nutrient starvation and elevated salinity results in a quick drop in viable cell counts with little change in total counts. A combination of elevated osmotic pressure (10x PBS), absence of nutrients, and exposure to visible light resulted in a drop in the number of cultivable cells to below our detection limits within 24 hours. Nutrient starvation with little to no osmotic stress (1x PBS; seawater) combined with light exposure resulted in a slower drop in the number of cultivable cells (Figure 3.2). After 24 hours of exposure to light and high osmotic stress (10x PBS) the number of viable cells fell below our limits of detection. High concentrations of cells plated might have yielded detectable numbers of cells that could form colonies.
Figure 3.2. Cell recovery under starvation conditions under elevated levels of salinity and bright light. Colony forming units were counted after 24 hour incubation in Petri dishes.

Microtiter well plate incubations of cells challenged by 10x PBS and bright light showed a 20% recovery of cells, all within 48 hours of incubation (Figure 3.3).

Figure 3.3. Percent wells exhibiting growth over an 8 day incubation period in a single cell format, after challenging cells with starvation, elevated osmotic pressure, and bright light.

*Incubation in diffusion chamber.* E. coli cells incubated for 1 week in diffusion chambers immersed into seawater showed a drop of two orders of magnitude in colony forming
units after 24-48 hours. Two week incubation in diffusion chambers showed drop of three orders of magnitude (Figure 3.4).

![Graph showing decrease in cfu over time in diffusion chamber](image)

Figure 3.4. Cells incubated in diffusion chambers in a sea water microcosm were removed plated after 0, 7, 14, and 21 days to determine the number of cells that could form colony-forming units (cfu) on Petri dishes after 24-48 hours.

The drop in cfu after incubation in diffusion chambers was confirmed in an additional experiment, in which the total count was monitored and proved stable over the time of incubation (Figure 3.5).
Figure 3.5. Cells incubated in diffusion chambers in a sea water microcosm were removed plated after 0, 18, and 21 days to determine the number of cells that could form colony-forming units (cfu) on Petri dishes after 24-48 hours. Total count was done in parallel at each time point.

Diffusion chambers sampled at finer time increments revealed that the drop in cultivable cells was most pronounced within the first 24 hours, and especially between 2 and 6 hours, and slowed down thereafter (Figures 3.6 and 3.7).

Figure 3.6. Cells incubated in diffusion chambers in a sea water microcosm were removed plated after 0, 1, 3, 11 and 18 days to determine the number of cells that could form colony-forming units (cfu) on Petri dishes after 24-48 hours.
Figure 3.7. Percent of cells capable of forming colonies within 24 hours that first spent 30 minutes, 1, 2, 4, 6 and 24 hours in a diffusion chamber.

**Growth recovery of cells over time from cultures incubated in diffusion chambers for 2 weeks.** *E. coli* cells in late stationary phase plated into microtiter plates in single and 10 cells per well format exhibited new instances of visible growth over a 2 week time period. At a single cell per well dilution, new wells exhibited growth at 24 hours, 5 and 8 days. At a 10 cell per well dilution, new wells exhibited growth at each time point observed during the 2 week incubation period. Petri dishes exhibited a comparatively low percentage of microscopically visible cells exhibiting growth and showed no additional growth over time (Figure 3.8).
Section 3.3.1.2 Starvation in late stationary/death phases

*Late stationary phase results in drop in viable counts.* After long term (5-12 month) incubation in a nutrient medium in sealed tubes, between 0.1% to 1% of cells remained readily cultivable (forming colonies within 24-48 hr). Once cell suspensions were moved to fresh LB for subculturing in the single cell format, new growth events occurred over
first 2 weeks, but ceased thereafter. Petri dishes showed a similar overall recovery but with a somewhat different kinetics (Figure 3.9).

![Figure 3.9. Percent cells from late stationary/death phase resuming growth in a single cell format (◆) and in Petri dishes (■).](image)

The 5, 10, and 20 cell/well format of subculturing showed similar trends (Figure 3.10).
Figure 3.10. Percent cells from late stationary/death phase resuming growth in the following formats: (a) 5 cell per well of a microtiter plate; (b) 10 cell per well; and (c) 20 cell per well.
**Anaerobic Incubation.** Incubation under anaerobic conditions resulted in no change over time in percent wells exhibiting growth beyond the initial recovery during the first week (Figure 3.11).

Figure 3.11. Percent wells inoculated with single cells in late stationary/death phase and exhibiting growth over time under anaerobic conditions.

However, after anaerobic conditions started to gradually change to microaerophilic, new instances of growth were apparent (Figure 3.12).

Figure 3.12. Percent wells inoculated with late stationary/death phase cells exhibiting growth after cells were incubated anaerobically, and then under an increasing concentration of oxygen.
Separation of late stationary phase cells by density centrifugation. Cells applied to a density gradient created with 60% Percoll resulted in two distinct bands (Figure 3.13). Plating did not reveal any difference in the number of cultivable cells between the two bands. This and other experiments (data not shown) failed to confirm results reported in the literature on differences in cultivability of cells from different bands.

![Figure 3.13. Two distinct bands present in density separation in a Percoll gradient of cells in late stationary phase.](image)

Section 3.3.2 Antibiotic treatment

Cultures treated with high concentrations of antibiotics (100x MIC) show new instances of growth beyond the typical 24-48 hour period required for *E. coli* growth. Cells that underwent treatment with a high dose of gentamicin initiated growth at various times over the 8 weeks of incubation. New colonies also appeared in Petri dish incubations but variability between dishes, and correspondingly large standard errors, do not allow to conclude whether or not there was a gradual recovery over time (Figure 3.14).
Figure 3.14. Awakening kinetics of dormant *E. coli*. (A) After antibiotic challenge, surviving individual cells of *E. coli* do not initiate growth simultaneously but start growing at different time points spread over two weeks of incubation. Gradual awakening is apparent in single cell experiments employing microtiter plates and in conventionally plated Petri dishes. Recovery at the end of two weeks is designated as ‘total recovery’ and assigned the 100% value; earlier data points presented as fractions of this value. The results represent data from three independent experiments, two of which employed gentamicin and one ofloxacin, all showing similar kinetics of growth initiation. (B) In a single growth experiment involving cells remaining after gentamicin treatment, we extended incubation period to two months, and observed a continuing appearance of new growths. For comparison, and to mimic conventional approaches to enumerate persisters, we incubated cells from the same source in Petri dishes. Note a much lower cell recovery in Petri dishes vs single-cell experiment in microtiter plates. Note that, in long-term incubation in Petri dishes, gradual awakening is not apparent, likely because of overgrowth by colonies developing early in incubation.

Cultures challenged with high doses of gentamicin or ofloxacin yielded variable cell recovery, but in all trials new instances of wells, inoculated with single cells and exhibiting growth, were observed beyond the usual 24 hour time period (Figure 3.15)
Cells challenged with antibiotics initiate growth continuously over eight weeks of incubation irrespective of growth phase at which cells were treated with an antibiotic, and irrespective of the antibiotic used. X-axis denotes whether gentamicin or ofloxacin was used to treat the cells and at which stage of growth cells were at the time of the application of the antibiotic.

Cultures treated with lower (10X MIC) dose of antibiotics show new instances of growth beyond the typical 24-48 hour period required for E. coli growth. Application of lower concentrations of gentamicin and ampicillin revealed the same pattern of new wells exhibiting growth beyond the typical time period (24 hours) usually required for E. coli to form colonies/turbidity. Cells challenged with gentamicin at a concentration of 5 µg/ml, showed new instances of growth over a five week incubation time in both both liquid and solid. In a corresponding set of Petri dishes no additional growth after the 24 hour time period was detectable (Figure 3.16).
Figure 3.16. Cells challenged with gentamicin (10X MIC) resume growth continuously after removal of the antibiotic. This is not observed in Petri dishes.

Experiments involving ampicillin showed a similar trend in single cell recovery over a 2 week incubation period after the antibiotic challenge, in incubation employing the single-cell format (Figure 3.17).

Figure 3.17. Cells challenged with ampicillin resume growth continuously after removal of the antibiotic.
Section 3.4 Discussion

The main goal of this part of the project is to establish whether or not \textit{E. coli} cells in non-growing states could resume growth, and if that growth resumption would show a pattern consistent with the scout model. Although a variety of methods have been designed to induce a large portion of \textit{E. coli} cells into a non-growing state (e.g., Colwell 2009; Oliver 2010; Mizunoe et al. 1999), debate is still ongoing over whether these cells are actually dormant, as opposed to dead/dying (Bogosian 2001; Bogosian et al. 1995; Nyström 2003). The overarching goal of this section was to find a reliable method by which cells of \textit{E. coli} could be put into a reversible dormant state, and observe their recovery from this state over time.

A number of studies have indicated that \textit{E. coli} can be put into the VBNC state, which our scout model equates with dormancy. These studies indicated that the VBNC state can be achieved through nutrient starvation, elevated salinity levels and exposure to visible light (Kjelleberg et al. 1987; Mizunoe et al. 1999; Munro et al. 1989; Na et al. 2006; Oliver 2005). I followed the procedures outlined in these studies, but failed to reproducibly confirm their results, and in most cases, even long term incubation showed little to no drop in the number of cultivable cells. Nutrient starvation in sterile water and in 1x PBS showed a slow drop in cultivable cells counts, contrary to the literature indicating the likelihood of a very quick drop in cultivable cell counts. Although it is possible that a slow drop in cultivable cells could indicate an entry of the cells of the culture into a state of dormancy, it is also possible I observed a slow dying off of the
population. An increase in osmotic pressure was achieved by incubating in 10x PBS. Combined with exposure to visible light, a drop in cultivability below the level of detection was observed after only 24 hours. Single cell incubations indicated that 20% of cells retained the ability to grow within 48 hours, indicating that 80% of cells from the original culture were either dead, or in a non-growing state. The single cell incubation revealed that there was no increase in cfu over an 8 day incubation period. This indicated that cells treated in this manner are not resuming growth consistent with the scout model. However, this may have multiple explanations. For example, it is not clear whether non-growing cells thus obtained are actually alive and viable, as opposed to dead or dying. Clearly, in the latter case attempts to observe their revival would be futile.

I attempted to obtain non-growing cells of *E. coli* that would be capable of growth resumption through incubating *E. coli* cultures under environmental conditions by placing cells into a diffusion chamber, and incubating the chamber while immersed into seawater. The rationale was that, upon exiting an animal’s digestive tract, an aquatic habitat is one likely encountered by at least some cells of *E. coli*, so that this species could have evolved a mechanism enabling survival in such habitat.

Incubation under these conditions revealed a substantial drop in cultivability after only a few hours. After six hours of incubation in the diffusion chamber, only 57% of the initially cultivable cells were capable of resuming growth within 48 hours. In one experiment, this proportion was as low as 10% after only 24 hours of incubation in a
diffusion chamber. This indicated that the majority of cells could have entered a non-growing state. Experimenting with such cells appeared to be one way to explore dormancy/exit from dormancy in *E. coli*. However, likely due to a high variability among the results of independent experiments, growth resumption proved rather inconclusive. While I did indeed observe resumption of cell growth beyond the expected (from *E. coli*) 24-48 hours, the length of time over which prolonged ‘awakening’ occurred (2 weeks) fell short of what could be good evidence of scout cells, which I arbitrarily judged as gradual recovery over weeks and months. I do not see these results as either confirming or rejecting the model: once again, the lack of gradual growth resumption of dormant cells can only serve as evidence against the scout model if the researcher knows in advance that the non-growing cells employed are indeed dormant and viable, as opposed to dead and dying, something that none of the experiments conducted could have assured.

Similarly, experimentation with the late stationary phase cells was not conclusive. Studying growth resumption in a single-cell format showed that some cells from the late stationary/death phase did initiate growth beyond the typical 24-48 hours. However, the total percent of wells exhibiting growth was quite low, around 1% of all the microscopically visible cells. The nature of the remaining 99% of cells remains unclear. Also unclear is whether a long-term incubation in a capped vial that initially contained the full-strength LB mimics conditions under which *E. coli* evolved its way of entering dormancy (if in fact it evolved in such a way at all).
I then hypothesized that perhaps a sudden exposure to oxygen of cells that spent months in a capped vial, under possibly anoxic or at least microaerophilic conditions, could have been so stressful and damaging that cell recovery would not mirror a pattern evolved in nature. Indeed, several studies have indicated that exposure of cultures kept at anaerobic or microaerobic conditions to oxygen may result in a negative effect on growth (Gregory et al. 1973). Accordingly, I subcultured cells from late stationary/death phases, in a single-cell format, under anaerobic conditions. The percent wells exhibiting growth was about 15 times higher compared to earlier experiment where cells were plated in the same manner under aerobic conditions indicating that exposure to oxygen did inhibit the ability of some cells to grow. However, no change in the percent wells exhibiting growth was observed for different lengths of incubation under anaerobic conditions, which can have more than one explanation, as discussed above.

My collective experiences from the experiments on inducing *E. coli* into a non-growing state suggest that, because we do not know how *E. coli* enters dormancy in nature, the possibility remains that all or some of my manipulations did not produce any significant number of truly dormant cells, and instead they damaged/killed the cells I was trying to induce into dormancy. I then explored a different strategy: instead of inducing a non-growing state, I attempted to enrich for pre-existing dormant cells.

An attractive and non-invasive way to achieve such an enrichment is an isopycnic density centrifugation, since dormant cells are likely to have a specific density different from that of their growing counterparts. Indeed, a study by Makinoshima et al. 2002
indicated that up to 15 discrete bands could be visualized in stationary phase cultures by density separation using Percoll. Another study indicated that density separation using Urografin resulted in the separation of culturable and nonculturable subpopulations of *E. coli* (Arana et al. 2008). Radioselectan was also able to separate subpopulations that exhibited differences in regard to ability to grow on standard media and ability to tolerate stress including nutrient starvation. Interestingly, these studies also showed that greater than 90% of cells were intact in both subpopulations as determined by a *BacLight*<sup>TM</sup> Live/Dead stain, and that the subpopulations showed similar levels of oxidative damage (Cuny et al., 2003; Desnues et al. 2005). I was able to recover two distinct bands using Percoll separation, however I did not detect any difference in cultivability between the two bands. After a series of extensive experiments, I had to admit that I was not able to reproduce the published results, and switched to another strategy based on killing growing cells.

Persister cells are known to be non-growing cells that survive antibiotic challenge via dormancy (Lewis 2010; Balaban et al. 2004). This suggests a simple way to enrich for dormant cells by treating a growing culture of *E. coli* with a high dose of an antibiotic. In fact, this way of enriching for dormant cells was the very first method I used, but the well known variability among replicates, which I also observed, made me explore alternative ways to put *E. coli* into dormancy (see above). Once the alternative ways failed, I returned to antibiotics use as a method to obtain dormant cells. In these experiments, I observed that, once cells remaining after the antibiotic treatment were placed in fresh medium, growth resumption occurred at least in some cells. The phenomenon of
persistence is well known (Lewis 2010), and such growth resumption per se was not surprising. Notable was the period of time over which this resumption continued. Regardless of the antibiotic used or its concentration, some of the challenged cells appeared to remain in non-growing state much beyond the typical (for *E. coli*) 24-48 hour incubation period, yet started growing thereafter. The time scale for what appears to be a gradual recovery is substantial, weeks to months (Figures 3.14-17), making it rather unlikely that the late comers were slowly repairing cells. I therefore argue that growth pattern of non-growing *E. coli* cells is consistent with the scout model, and follows the predictions of the latter.
Section 4. Dormant cells of *Mycobacterium smegmatis* show a linear recovery over time, indicating randomness of their awakening.

Section 4.1 Introduction

Modern medicine has resulted in effective treatment of many diseases, yet latent infections continue to plague patients worldwide. Latent infections are defined as the physical presence of the pathogen without the presence of symptoms. While the exact details of how bacteria survive in this latent phase is unclear, a logical explanation, and one that is commonly accepted, is that they are in dormant state (Chao et al. 2010). Through dormancy, a number of infectious agents can cause lifelong problems for the patient. As most treatments today target actively growing cells, it is of particular interest how the pathogen exits this state, and reinfects. This knowledge could be applied to developing treatments of dormant pathogens, or conversely to finding a way to activate dormant cells and then using current treatments to clear infection. In this section, I will examine how individual cells of *Mycobacterium smegmatis* (a model organism for the pathogen *Mycobacterium tuberculosis* (Reyart et al. 2001)) resume growth following entry into dormancy, and examine if the pattern observed conforms to the predictions of the scout model.

Induction of dormancy in a *M. tuberculosis* culture has been established via the Wayne model of slow oxygen depletion. This method of dormancy induction has also been applied to *M. smegmatis*, a non-pathogenic, faster growing relative of *M. tuberculosis*. In the specific case of dormancy induction via the Wayne model, the mechanisms by
which both *M. smegmatis* and *M. tuberculosis* enter dormancy seem to be similar (Dick et al. 1998; Tyagi et al. 2002).

In this section, I will show that dormant cells (induced via the Wayne model) of *M. smegmatis* resume growth in a pattern consistent with the predictions of the scout model.

**Section 4.2 Materials and Methods**

*Strains, medium and growth conditions.* *M. smegmatis* strain mc\(^2\)155 was kindly provided by the Lewis Lab (Northeastern University). Cells were grown in Difco Middlebrook 7H9 Broth (Benton-Dickson, Franklin Lakes, NJ) supplemented with bovine serum albumin, dextrose, NaCl, catalase and 20% tween 80 (hereafter referred to as 7H9 broth). Cells were grown for 48 hours with shaking at 37°C to mid-stationary phase.

*Dormancy induction.* Dormancy induction was achieved via the Wayne Method (Dick et al. 1998; Wayne et al. 1996). One µl loops of mid-stationary phase culture were inoculated into 17 ml of supplemented 7H9 broth in 20 x 125 mm glass screw cap test tubes (Kimble Chase, Vineland, NJ). Oxygen depletion was monitored in control tubes via the addition of methylene blue at a concentration of 1.5 µl/ml. Absence of blue color indicated that oxygen was depleted in control tubes. Viable cell counts were made by
plating onto 7H9 agar and counting colony forming units after 48 hours and 1 week of incubation.

*Regrowth of dormant cells.* Cell suspensions obtained via the Wayne Method were removed from the incubator and sonicated using a Sonics Vibra Cell Ultrasonic Processor (Sonics, Newton, CT) two times for 20 seconds at the 40 amplitude setting to break up cell aggregates. Absence of cell aggregates was confirmed microscopically.

Following general recommendations by Hobbie et al. (1977), subsamples for microbial enumeration were prepared by serially diluting the cell suspensions in sterile water and fixed with 4% formaldehyde (Sigma, St. Louis, MO). Cells were stained by mixing 100 µl of a cell dilution, 20 µl of DAPI (4′,6-diamidino-2-phenylindole) stain, and 2 ml of DNA grade water. After 10 minutes of staining in the dark, cells were collected on a 0.2-µm pore-size polycarbonate filter (Millipore, Billerica, MA). Filters were washed twice with DNA grade water, and mounted on standard glass slides. Filters were viewed at 1000x using a Zeiss Aksioskop 50 compound microscope equipped for epifluorescence. A minimum of 16 fields and 250 cells were counted for each preparation.

Suspensions of cells obtained via the Wayne Method were mixed with 7H9 broth to an approximate density of 1, 10, 25, 50 or 100 cells per 50 µl. A 12-channel micropipetter was used to distribute 50 µl aliquots of thus obtained dilutions over 384-well microtiter plates. In each plate, 360 wells received the above aliquots, whereas the remaining 24
wells were filled with sterile medium as a negative control. The loaded plates were sealed with Parafilm and incubated in humidifying chambers at 37°C.

Plates were inspected at 48 hours, 5 days, 1 week, and weekly thereafter for growth visible to the naked eye. Wells exhibiting growth were marked and the time of growth appearance recorded.

Subculture experiments. Wells that exhibited growth early (72 hours) and late (2 weeks) during the incubation of the microtiter plates were subcultured. Grown material was removed with sterile syringe, and microbial cells were enumerated via DAPI staining (as described above). Cells were diluted with 7H9 broth to an approximate density of one or ten cells per 50 µl. A 12-channel micropipetter was used to add 50 µl aliquots of thus obtained cell suspension to 360 wells in a 384-well microtiter plate for each sample. The top row (24 wells) was filled with sterile medium as a negative control. The plates were sealed with Parafilm, incubated in humidifying chambers at 37°C, and observed for growth in the manner described above at 48 hours and 1 week.

Section 4.3 Results

Wayne Model dormancy induction results in majority of microscopically visible cells being in a non-growing state on 7H9 medium. Cells of M. smegmatis were treated via the Wayne Model of oxygen depletion. Control cultures containing methylene blue had no visible blue color after 180 hours incubation. After 300 hours of incubation, cell
enumeration showed *M. smegmatis* density of $10^7$ cells per ml. At 48 hours after plating these cells on fresh medium, the number of colony forming units indicated $10^4$ actively growing cells per ml of cell suspension, or 0.1% of microscopically visible cells. This is consistent with the reported applications of Wayne Method.

*Individual cells of dormant M. smegmatis resume growth in a linear fashion over an extended incubation period.* Dormant cells of *M. smegmatis* inoculated into microtiter plates at concentrations of 1, 10, 25, 50 and 100 cells per well all showed recovery beyond the typical time *M. smegmatis* forms visible growth (48-72 hours). In a single-cell format, accumulation of wells with visible growth was nearly linear in multiple trials (Figure 4.1).

![Graph showing growth over time](image)

**Figure 4.1.** Two independent long-term experiments show that dormant cells of *M. smegmatis* initiate growth at apparently random time points, with cumulative growth curve exhibiting no sign of leveling off even after 3 months of incubation.
Experiments in 10 cells per well format also showed a steady recovery over the incubation period, with new instances of growth seen as late as 91 days incubation (see figure 4.2)

Figure 4.2. Two independent long-term experiments show that dormant cells of *M. smegmatis* initiate growth at apparently random time points, with cumulative growth curve exhibiting no sign of leveling off even after 3 months of incubation.

Other formats (25, 50, and 100 cells/well) all showed additional wells exhibiting growth after the 48-72 hour time point (data not shown).

*Subculturing microbial growths that appeared early and late during regrowth experiment showed a similar pattern.* To check if microbial cultures that took longer to grow in the long-term experiments were populated by inherently slow growing variants, I subcultured these cultures in a single and 10 cell per well format. Growth of these subcultures was similar irrespective of the initial time to grow. Although more wells
exhibited growth in the sample from the earlier time point, both plates contained cells that displayed visible growth within 72 hours (Figure 4.3).

![Graph showing percent wells exhibiting growth over time]

Figure 4.3. Cultures that appeared early vs late in the long-term experiment on recovery of dormant *M. smegmatis*, show little difference in the secondary growth rate upon subculturing, as shown by two trials, in one- and 10 cell/well formats.

**Section 4.4 Discussion**

These experiments were undertaken to examine if individual dormant cells of *M. smegmatis* would exhibit a pattern of awakening consistent with the scout model.

Dormancy induction was achieved via the Wayne model and resulted in a cell suspension in which 99.9% of the cells appeared non-growing (i.e., were unable to form a colony over a period of time a typical growing cell of *M. smegmatis* does (48-72 hr)). As expected, upon subculturing such cells, I observed a low (0.01%) initial recovery within the first 48-72 hr of incubation. However, the recovery increased with time, in a remarkably linear fashion, over a period of several months.
It is possible that this linear recovery was a product of genetic differences among the dormant cells such that some were faster growers, and others were slower growers. Therefore, it was important to subculture microbial biomass that appeared at different time points in the initial experiments, and to determine the secondary growth rates. If they were consistent with the rate of initial growth, and thus widely different, it would indicate inherent differences among the variants recovered with respect to their division time. However, subculture experiments showed that secondary growth rates were similar among different isolates, arguing against the idea of slow- and fast growing variants of *M. smegmatis*. The simplest explanation of the observed growth pattern is that dormant cells of *M. smegmatis* woke up in random fashion. This is important because *M. smegmatis* is a model organism for studying tuberculosis, and if growth behavior of *M. tuberculosis* also conforms to the scout model, it may have implications for the control of the disease. Indeed, if the molecular mechanism of scout formation was known, one could devise a method for awakening all dormant cells, which, upon resumption of growth, would become vulnerable to the existing antibiotics.

In light of this, and knowing there were numerous cells in Wayne-treated cultures that were capable of spontaneously initiating growth, I became interested in inducing growth of such viable but dormant cells of *M. smegmatis*. The relevant experiments are described in Section 5.
Section 5. Dormant cells of *Mycobacterium smegmatis* can be induced to grow by actively growing kin cultures.

Section 5.1 Introduction

The scout model postulates that, at least in some species, proliferating scouts induce the remaining dormant kin cells to grow (Figure 1.1). Indeed, there is growing evidence that non-growing cells start dividing in the presence of the cell-free supernatant of actively growing kin cultures (Mizunoe et al. 1999; Oliver et al. 1995; Oliver 2004; Roth 1988). For *M. tuberculosis* there is evidence that non-growing cells can be induced to grow by the application of resuscitation promoting factor (rpf), which was isolated from *Micrococcus luteus* (Mukamolova et al. 2002; Kana et al. 2008). Rpf is a muralytic enzyme that cuts peptidoglycan (Mukamolova et al. 2006). Another study reported a similar phenomenon in *Bacillus subtilis*, in which muropeptides fragments were released by actively growing cells and induced germination of *Bacillus* spores (Shah et al. 2008). Additionally, Nichols et al. were able to isolate, from actively growing cultures of an environmental species, a small peptide that acted as signal to induce growth in non-growing cells of this species (Nichols et al. 2008).

Therefore, the postulated component has a significant support in the literature. However, considering the medical importance of one of the model species used here, *M. smegmatis*, it would be interesting to check if in that species, and by extension in other Mycobacteria, growth induction of dormant cells was also part of their life cycle. This is particularly important because the majority of treatments available today for tuberculosis target actively growing cells (Chao et al. 2010). This makes dormant cells
essentially untreatable by traditional means and may contribute to the long term latency of tuberculosis. In principle, if dormant cells were induced to grow, they could be eliminated by the available antibiotics, which is important given that ⅓ of the world’s population may harbor a tuberculosis infection, and of these infections 90% are latent (Wayne 1996; Chao et al. 2010; others).

In this section, I show that, in accordance with the scout model, growing cells of \textit{M. smegmatis} can induce dormant cells of this species to grow, and present preliminary data on the nature of the inducing activity. This was achieved in collaboration with Jason Crawford and Jon Clardy, Harvard Medical School. Bioassays were conducted at Northeastern, while separation analyses performed in J. Clardy’s lab.

\textbf{Section 5.2 Materials and Methods}

\textit{Strains, medium and growth conditions}. \textit{Mycobacterium smegmatis} strain mc²155 was kindly provided by the Lewis Lab (Northeastern University).

Cells were grown, enumerated and induced into non-growing state as described in Section 4. In brief, cells were grown in 7H9 broth for 48 hours with shaking at 37°C to mid-stationary phase.

Dormancy induction was achieved via the Wayne Method (Dick et al. 1998; Wayne et al. 1996). One µl loops of mid-stationary phase culture were inoculated into 17 ml of 7H9
broth in 20 x 125 mm glass screw cap test tubes (Kimble Chase, Vineland, NJ).

Oxygen depletion was monitored in control tubes via the addition of methylene blue at a concentration of 1.5 µl/ml. Absence of blue color indicated that oxygen was depleted in control tubes. Viable cell counts were made by plating onto 7H9 agar and counting colony forming units after 48 hours and 1 week of incubation. Cell suspensions obtained via the Wayne Method were removed from the incubator and sonicated using a Sonics Vibra Cell Ultrasonic Processor (Sonics, Newton, CT) two times for 20 seconds at the 40 amplitude setting to break up cell aggregates. Absence of cell aggregates was confirmed microscopically.

Following general recommendations by Hobbie et al. (1977), subsamples for microbial enumeration were prepared by serially diluting the cell suspensions in sterile water and fixed with 4% formaldehyde (Sigma, St. Louis, MO). Cells were stained by mixing 100 µl of a cell dilution, 20 µl of DAPI (4’,6-diamidino-2-phenylindole), and 2 ml of DNA grade water. After 10 minutes of staining in the dark, cells were collected on a 0.2 µm pore-size polycarbonate filter (Millipore, Billerica, MA). Filters were washed twice with DNA grade water, and mounted on standard glass slides. Filters were viewed at 1000x using a Zeiss Aksioskop 50 compound microscope equipped for epifluorescence. A minimum of 16 fields and 250 cells were counted for each preparation.

*Small compound library.* Twenty-seven compounds were selected for testing on their ability to induce dormant *M. smegmatis* to grow based on their likelihood to be involved in bacteria signaling processes. All of the compounds were selected based on evidence
in the literature that they were involved in induction of growth in bacteria. These compounds are listed in Table 5.1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Compound</th>
<th>Stock Concentration</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>Schizokinen</td>
<td>0.03 mg/ml</td>
<td>0.005 mg/ml</td>
</tr>
<tr>
<td>L2</td>
<td>Enterobactin</td>
<td>0.33 mg/ml</td>
<td>0.055 mg/ml</td>
</tr>
<tr>
<td>L3</td>
<td>Yersiniabactin</td>
<td>0.33 mg/ml</td>
<td>0.055 mg/ml</td>
</tr>
<tr>
<td>L4</td>
<td>Desferrioxamine B</td>
<td>0.03 mg/ml</td>
<td>0.005 mg/ml</td>
</tr>
<tr>
<td>L5</td>
<td>Iron II sulfate</td>
<td>240 µM</td>
<td>40 µM</td>
</tr>
<tr>
<td>L6</td>
<td>Vitamin (One A Day Advanced™)</td>
<td>0.6 mg/ml</td>
<td>0.1 mg/ml</td>
</tr>
<tr>
<td>L7</td>
<td>D-tryrosine</td>
<td>6 mM</td>
<td>&lt;1 mM</td>
</tr>
<tr>
<td>L8</td>
<td>D-amino acid mix (D-valine, D-histidine, D-isoleucine, D-proline, D-tyrosine, D-aspartic acid)</td>
<td>2 mg each amino acid in 5 ml water</td>
<td>2 mg each amino acid in 5 ml water</td>
</tr>
<tr>
<td>L9</td>
<td>D-isoleucine</td>
<td>6 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>L10</td>
<td>L-proline</td>
<td>300 mM</td>
<td>50 mM</td>
</tr>
<tr>
<td>L11</td>
<td>L-proline</td>
<td>6 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>L12</td>
<td>Casamino acids</td>
<td>0.06% v/v</td>
<td>0.1% v/v</td>
</tr>
<tr>
<td>L13</td>
<td>Betaine</td>
<td>12 mM</td>
<td>2 mM</td>
</tr>
<tr>
<td>L14</td>
<td>Carnitine</td>
<td>12 mM</td>
<td>2 mM</td>
</tr>
<tr>
<td>L15</td>
<td>Riboflavin</td>
<td>600 µM</td>
<td>100 µM</td>
</tr>
<tr>
<td>L16</td>
<td>Nicotinic acid</td>
<td>600 µM</td>
<td>100 µM</td>
</tr>
<tr>
<td>L17</td>
<td>Thiamine</td>
<td>600 µM</td>
<td>100 µM</td>
</tr>
<tr>
<td>L18</td>
<td>Glucose</td>
<td>0.6% m/v</td>
<td>0.1% m/v</td>
</tr>
<tr>
<td>L19</td>
<td>Glutathione</td>
<td>6 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>Name</td>
<td>Compound</td>
<td>Stock Concentration</td>
<td>Final Concentration</td>
</tr>
<tr>
<td>------</td>
<td>---------------------------------</td>
<td>---------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>L20</td>
<td>Chorismic acid</td>
<td>1.2 mM</td>
<td>200 µM</td>
</tr>
<tr>
<td>L21</td>
<td>N-acteyl-glucosamine</td>
<td>6 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>L22</td>
<td>Cholesterol</td>
<td>600 µM</td>
<td>&lt;100 µM</td>
</tr>
<tr>
<td>L23</td>
<td>3,4-dimethoxycinnamic acid</td>
<td>600 µM</td>
<td>&lt;100 µM</td>
</tr>
<tr>
<td>L24</td>
<td>Vanillin</td>
<td>600 µM</td>
<td>100 µM</td>
</tr>
<tr>
<td>L25</td>
<td>$^{13}$C-Palmitic acid</td>
<td>6 mM</td>
<td>&lt;1 mM</td>
</tr>
<tr>
<td>L26</td>
<td>Sodium acetate, pH 5.2</td>
<td>300 mM</td>
<td>50 mM</td>
</tr>
<tr>
<td>L27</td>
<td>Sodium acetate, pH ~7</td>
<td>300 mM</td>
<td>50 mM</td>
</tr>
</tbody>
</table>

Table 5.1. List of chemical compounds tested in the *M. smegmatis* growth induction bioassays.

*Preparation of spent medium.* Five hundred ml of 7H9 broth were inoculated with 10-µl loops of mid-stationary phase *M. smegmatis*. Cultures were grown with shaking at 37°C for either 18 hours (mid-exponential phase) or 48 hours (mid-stationary phase). Cultures were taken and spun at 4,000 rpm (3,313 g) in 50 ml tubes in a Sorvall ST-H750 rotor swing out bucket rotor using a Sorvall Super T21 Centrifuge. Supernatant was removed and filtered through a 0.2 micron membrane in a Nalgene filter tower (Nalge Nunc, Rochester, NY).

*Extracts of spent medium.* Extracts of spent medium samples from both mid-exponential and stationary phase were prepared as described in Table 5.2.
<table>
<thead>
<tr>
<th>Name</th>
<th>Extract</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA</td>
<td>Ethyl Acetate (EtOAc) extract, mid-exponential phase</td>
<td>100 ml of mid-exponential phase spent medium was divided into four 50 ml tubes. The organic layer was pooled, then dried. The dried material was resuspended in 1 ml of water.</td>
</tr>
<tr>
<td>MB</td>
<td>XAD-2 (resin), mid-exponential phase</td>
<td>XAD-2 (Supelco) solid-phase resin was added to 100 ml of mid-exponential phase spent medium and stirred continuously for 2-3 hours. This sample as applied to a glass column, washed with water, then eluted with 60 ml of 100% methanol. The sample was resuspended in 2 ml of water and 2 ml of dimethyl sulfoxide (DMSO).</td>
</tr>
<tr>
<td>MC</td>
<td>Methanol extract, mid-exponential phase</td>
<td>100 ml of mid-exponential phase spent medium was divided into four 40 ml vials. The material was dried using a speed vacuum concentrator, then extracted using 50 ml of 100% methanol and dried again using the speed vacuum concentrator. The dried material was resuspended in 2 ml of water.</td>
</tr>
<tr>
<td>MD</td>
<td>Concentrated water-soluble component of the spent medium, mid-exponential phase</td>
<td>50 ml of mid-exponential phase spent medium extract was dried using a speed vacuum concentrator, then resuspended in 3.5 ml of water.</td>
</tr>
<tr>
<td>ME</td>
<td>Concentrated macromolecules from the spent medium, mid-exponential phase</td>
<td>22 ml of mid-exponential phase spent medium extract was concentrated by ultrafiltration using an amicon 5000 nominal molecular weight cutoff ultrafiltration system. 1.3 ml of sample was collected and frozen in liquid nitrogen.</td>
</tr>
<tr>
<td>SA</td>
<td>Ethyl Acetate (EtOAc) extract, stationary phase</td>
<td>100 ml of stationary phase spent medium was divided into four 50 ml tubes. The organic layer was pooled, then dried. The dried material was resuspended in 1 ml of water.</td>
</tr>
<tr>
<td>SB</td>
<td>XAD-2 (resin) extract, stationary phase</td>
<td>XAD-2 (Supelco) solid-phase resin was added to 100 ml of mid-exponential phase spent medium and stirred continuously for 2-3 hours. This sample as applied to a glass column, washed with water, then eluted with 60 ml of 100% methanol. The sample was resuspended in 2 ml of water and 2 ml of dimethyl sulfoxide (DMSO).</td>
</tr>
<tr>
<td>Name</td>
<td>Extract</td>
<td>Method</td>
</tr>
<tr>
<td>------</td>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>SC</td>
<td>Methanol extract, stationary phase</td>
<td>100 ml of stationary phase spent medium was divided into four 40 ml vials. The material was dried using a speed vacuum concentrator, then extracted using 50 ml of 100% methanol and dried again using the speed vacuum concentrator. The dried material was then resuspended in 2 ml of water.</td>
</tr>
<tr>
<td>SD</td>
<td>Concentrated water-soluble component of the spent medium, stationary phase</td>
<td>50 ml of stationary phase spent medium extract was dried using a speed vacuum concentrator, then resuspended in 3.5 ml of water.</td>
</tr>
<tr>
<td>SE</td>
<td>Concentrated macromolecules from the spent medium, mid-exponential phase</td>
<td>22 ml of stationary phase spent medium extract was concentrated by ultrafiltration using an amicon 5000 nominal molecular weight cutoff ultrafiltration system. 1.3 ml of sample was collected via this ultrafiltration and frozen in liquid nitrogen.</td>
</tr>
</tbody>
</table>

Table 5.2. Types of *M. smegmatis* spent medium extracts used in the *M. smegmatis* growth induction bioassays.

*Methanol fractionation.* Spent medium samples were extracted with ethyl acetate, dried and resuspended as described above. Resuspended material was applied to a SepPak column (Waters Corporation, Milford, MA) and the flow through (FT) fraction that did not bind to the column was collected. Eluted compounds were collected using increasing concentrations of methanol (20, 40, 60, 80 and 100% MeOH). A final wash was carried out with 50% methanol and 50% dichloromethane.

*RP-Amide separation of methanol fractions.* 0.6 ml of the active fraction (FT fraction) was separated over a semi-preparative RP-amide reversed phase column (Phenomenex, Torrance, CA) using a gradient of 0.1% formic acid and 2% MeOH to 8 minutes, 20% MeOH to 30 minutes, 80% MeOH to 40 minutes, 80% MeOH held to 50 minutes and 100% MeOH to 55 minutes (2 ml/min). A total of 16 fractions with
detectable peaks were obtained using a diode array detector at 210, 254, and 280 nm. Individual fractions were resuspended in 0.5 ml sterile water.

Chemical analysis of fractions. Analysis of the active fraction was done using a diode array detector at 280 nm. The +1 ion of the fraction was selected by Liquid Chromatography/Mass Spectrometry (LC/MS). The UV visible spectra was compared to the UV-visible database (assembled in Bill Fenical’s lab at Scripps Research Institute of Oceanography) and also to authentic standard of 3-acetylpyridine using a diode array detector.

Phenylethylamine screen. 2-Phenylethylamine hydrochloride (Sigma, St. Louis, MO) powder was dissolved into sterile water then filtered through a 0.2 micron filter attached to a sterile syringe and serially diluted. Dilutions were added to 7H9 broth containing dormant cells at a concentration of 50 cells per volume of well. Concentrations of 2-phenylethylamine hydrochloride used were 1nM, 5nM, 20nM, 100nM, 500nM, 2µM, 10µM, 250µM, 500µM, 1mM, 2.5mM, and 10mM. Plates were incubated as described above and observed for growth at 72 hours and 1 week.

Bioassay. Standard bioassays were not suitable for testing the above compounds, extracts and fractions because, in batch cultures, growth induction of dormant cells would be masked by randomly awakening scouts. The single cell method employed in earlier experiments solves this problem, but given the rate of scout awakening and expected number of cells that could be induced to grow, this single-cell method would
be impractical by requiring hundreds of microtiter plates. Based on experiments involving various cell concentrations (see Section), I was able to determine that a cell concentration of 50 cells per well was a good compromise between the requirements (i) to have as many growth induction events per plate as possible (to minimize the number of plates needed), and (ii) to have a sufficiently low number of stochastically awakening scouts, which would be false positives.

Thus, compounds, extracts and fractions were tested by the application of 10 µl of each compound, extract or fraction to multiple wells of a 384 well plate containing 50 dormant *M. smegmatis* cells per well. Visible growth was recorded at 48 hours and 1 week, and the number of wells exhibiting growth was compared to control wells that received no compound.

**Section 5.3 Results**

*Small molecule library and spent medium extract screen.* A library of 27 compounds (Table 5.1) and 12 extracts (Table 5.2) from *M. smegmatis* mid-exponential and stationary phase spent medium were applied to dormant cultures of *M. smegmatis*.

All oxygen-depleted cultures of *M. smegmatis* produced a certain level of scouts that grew regardless of whether the growth inducing compound was/was not present (see Section 4). These were controlled for by measuring the number of growth events in the non-amended wells. If amended wells exhibited growth statistically different from
control, the difference was attributed to the compound/extract/fraction present in such wells. A 50 dormant cell per well appeared a good compromise between keeping the growth in control (false positives) sufficiently low to detect a difference introduced by the amendment, doing so using a reasonable number of microtiter plates.

The addition of sodium acetate with a pH of 5.2 (compound L26) resulted in no wells exhibiting growth, suggesting growth suppression. This was likely due to low pH value, since addition of sodium acetate with a pH of ~7, while showing a lower percentage of wells exhibiting growth than the control at 48 hours, resulted in the number of growth events indistinguishable from control by Day 7 (Figure 5.2).

Addition of ethyl acetate (EtOAc) extract of the mid exponential phase spent medium (MA), and also of XAD-2 (resin) extract of stationary phase spent medium (SB), appeared to have the strongest growth inducing effect (Figure 5.2).
Figure 5.1. Growth recovery of dormant *M. smegmatis* as influenced by the addition of compounds 1 through 12 from the small compound library, vs control (-).

Figure 5.2. Growth recovery of dormant *M. smegmatis* as influenced by the addition of compounds 13 through 27 from the small compound library, and addition of spent medium extracts of mid-exponential phase (M) and stationary phase (S) *M. smegmatis*, vs control (-).

**Screen of methanol fractions of EtOAc extract of mid-exponential phase of *M. smegmatis spent medium***. The EtOAc extract of the *M. smegmatis* mid-exponential phase spent medium was separated based on these compounds' polarity resulting in seven fractions. These fractions were applied to dormant cells of *M. smegmatis* in the same format as above, and growth was scored at 72 hours, 5 days, 1 week and 2
weeks. After one week of incubation, four treatments exhibited a statistically significant induction of growth: filtered and unfiltered EtOAc extracts, as well as the 100% Methanol Fraction and the Flow Through (FT) Fraction. Over the course of a two-week incubation time period, wells with the 100% Methanol Fraction and FT Fraction added showed different patterns of appearance of wells exhibiting growth (Figure 5.3).

The 100% Methanol Fraction showed, at the 72-hour time point, the largest increase in the percent wells exhibiting growth compared to control. Visually, growth in wells amended with this fraction appeared denser than in other wells. However, over the two-week incubation period, the difference between the number of wells exhibiting growth in the wells that received 100% Methanol Fraction, and those that received no treatment, became smaller. The pattern of growth in the presence of the FT Fraction was opposite: little difference from control at 72 hours, but statistically significant induction at Day 7 (Figure 5.3).
Figure 5.3. Growth of dormant *M. smegmatis* in wells amended with the 100% Methanol Fraction (●) and Flow-Through (FT) Fraction (■), presented as % increase over the control values.

**HPLC of FT Fraction.** We further separated the FT Fraction using the High Performance Liquid Chromatography (HPLC) with RP-amide separation into 55 fractions. UV spectrum detection identified 16 fractions with detectable peaks (Figure 5.4), which were bioassayed (see below). The fractions were named according to the minute at which they were recovered (see Figure 5.4). Fractions tested were named 7, 8, 9, 10, 11, 12, 17, 19, 22, 23, 26, 42, 44, 46, 48 and 53.
Bioassay of fractions of FT fraction. Addition of Fraction 17 showed 109% more wells exhibiting growth compared to the baseline (the percent wells exhibiting growth in the control). Addition of Fraction 42 showed an increase of 127% more wells exhibiting growth compared to the baseline, but it was not statistically significant. Addition of Fraction 22 showed a decrease of 48% in the number of wells exhibiting growth compared to the baseline (Figure 5.5).
Chemical analysis of Fraction 17. As Fraction 17 showed growth induction of dormant cells of *M. smegmatis*, the chemical composition of Fraction 17 was further investigated. The UV spectrum analysis of Fraction 17 did not provide a match to known compounds in the available databases. Liquid Chromatography/Mass Spectrometry (LC/MS) revealed that there was a major compound and some minor compounds present (see figure 5.6).
UV visible chromophore for the major compound was similar to that of functionalized pyridine, indicating that 3-acteylpyridine might be similar to the major compound. The latter is slightly more polar than 3-acteylpyridine based on the shift in maximums in the UV visible comparison and based on the fact that it migrated faster than 3-acteylpyridine (Figure 5.7).

![Figure 5.7. UV visible spectra of fraction 17 (---) and 3-acteylpyridine (---).](image)

**HR-MS of major compound in FT Fraction.** The major compound in the FT Fraction of the EtOAc extract of mid-exponential phase spent medium was found to have the chemical formula C₈H₁₂N by High Resolution Mass Spectrometry (HR-MS). The possible candidates include alkyl-aniline, alkyl-pyridine and phenylethylamine.

**Phenylethylamine screen.** Phenylethylamine was identified as one of the potential compounds in the FT Fraction (see above), and we decided to test the commercially available phenylethylamine in various concentrations in the *M. smegmatis* growth induction bioassay. Addition of 1mM of phenylethylamine showed a 54% increase in
wells exhibiting growth compared to the control. Addition of 10mM of phenylethylamine showed a 93% decrease in wells exhibiting growth compared to the control (see figure 5.8).

![Figure 5.8. Growth of dormant M. smegmatis in wells amended with 2-phenylethylamine.](image)

Addition of a wider range of concentrations showed a range of increase in wells exhibiting growth compared to the control from 5% increase up to 32% increase. The largest increase in percent wells exhibiting growth (32%) was seen with the addition of 10 µM of phenylethylamine (Figure 5.9).
Section 5.4 Discussion

The main goal of this part of my project was to provide support for one specific aspect of the scout model, namely that a growing culture could induce dormant kin cells to grow. Recent findings suggest that this may indeed be happening in environmental strains (Nichols et al. 2008), *M. luteus* and *M. tuberculosis* (Mukamolova et al. 2002; Kana et al. 2008), and *B. subtilis* (Shah et al. 2008).

Here I test whether a similar phenomenon exists in a medically important species of *M. smegmatis*. Based on earlier experiments I knew that, in an oxygen-depleted culture *M. smegmatis*, a pool of cells existed that, while in principle capable of growth, would not initiate division immediately upon exposure to appropriate nutrients (Section 4, Figure 4.1-2). Here I attempt to induce growth of these cells using supernatants of a growing culture of *M. smegmatis*, and conduct bioassay-guided fractionation of such
supernatants to follow the chemical nature of the activity involved. The major challenge is to distinguish growth induced by a factor applied from spontaneous initiation of growth by scout cells determined in Section 4 (Figure 4.1-2). The latter is unavoidable, and from the point of growth induction study, represents false positives. The number of these false positives can be minimized in a single-cell format of *M. smegmatis* cultivation, but in this format the number of induced growths may prove too small to detect unless a very large number of cells (and thus microtiter plates) are employed in each bioassay. The number of wells showing induced growth grows as the number of cells inoculated into each well grows, but this has its own limitation since the number of false positives will grow as well. As a result of a substantial preliminary experimentation, I determined that inoculating 50 dormant *M. smegmatis* cells per well represented a reasonable compromise: the number of scouts determined in Section 4 would explain approximately 30% wells showing growth after 3 days, leaving the remaining 70% wells to show growth induction.

My first attempt to induce growth involved testing the several small molecules selected for their roles in signaling and/or growth induction (Beumer et al. 1994; Crawford et al. 2009; D'onofrio et al. 2010; Gross et al. 1989; Lakaye et al. 2004; Lam et al. 2009; Lankford et al. 1966; Melchers et al. 2006; Özcan et al. 2007; Rajamani et al. 2008; Sonenschein 2007). No growth induction was observed, and the only compound that appeared to cause any noticeable difference from the control was sodium acetate (pH=5.2), which showed complete suppression of any growth. Most likely, this suppression of growth was due to the low pH.
In parallel, I tested spent medium extracts from actively growing cultures of *M. smegmatis* at mid-exponential and stationary phase on their potential role as growth inducers. A number of extraction methods were selected in order to get a range of polar and non-polar metabolite concentrations. I observed that addition of an EtOAc extract of mid-exponential phase showed signs of growth induction of dormant *M. smegmatis* (Figure 5.2). It stood to reason to further fractionate the EtOAc mid-exponential phase extract, via methanol fractionation, and test the fractions on their ability to induce growth of dormant *M. smegmatis*. The methanol fractionation of the EtOAc extract resulted in 7 fractions of varying polarity, and two of them, the 100% Methanol Fraction and the FT Fraction, appeared to induce a statistically significant number of dormant *M. smegmatis* cells to grow (Figure 5.3).

The 100% Methanol Fraction was composed of relatively non-polar compounds. It also appeared ‘soapy’ indicating the presence of some lipid-like compounds. LC/MS of the the 100% Methanol Fraction showed a related series of compounds with molecular weights ranging from 716.5 to 818.6. They matched two known compounds in the available database- the ferric and non-ferric forms of carboxymycobactins. In the future, it would be interesting to test the direct addition of carboxymycobactins to see if growth induction would occur. Additionally, it would be quite interesting to see if the ferric or non-ferric forms affect the growth induction.
The addition of the 100% Methanol Fraction resulted in an increase of growth that initially seemed quite high compared to the control. As the incubation progressed, the recovery of the control wells compared to that amended with 100% Methanol Fraction came closer. Bioassay showed that the growth that appeared in the presence of 100% Methanol Fraction was quite different than that of the control wells- it was considerably denser. Because by the end of the incubation period the number of amended wells showing growth was indistinguishable from control, it seems that this fraction did not induce any additional cells to grow, instead increasing growth rates of scouts so that more of them showed visible growth at the very first time point.

The Flow Through Fraction (FT) was composed of very polar compounds, such as sugars, carbohydrates, osmolytes, and short chain fatty acids. LC/MS of the FT Fraction showed 5 major compounds, and many minor products (Figure 5.6). In the bioassay, addition of the FT Fraction resulted in nearly double the amount of wells showing growth vs the control (Figure 5.3). Interestingly, the increase was only detectable after 5 days incubation, and peaked around 7 days of incubation. Unlike the addition of the 100% methanol fraction, the wells that did show growth all looked similar to those seen in the control (i.e., there was no increase in density of culture within the wells). The FT Fraction seemed to be the most likely candidate for the growth induction compound from the EtOAc extract, warranting further experimentation with HPLC fractions of FT.
Separation of the flow through (FT) fraction showed that addition of the fraction recovered at 17 minutes (Fraction 17) showed 109% increase in wells exhibiting growth compared to the control. The fraction recovered at 22 minutes (Fraction 22) showed a 47% decrease in wells exhibiting growth compared to the control. Both effects are statistically significant. Therefore, at least one fraction of the *M. smegmatis* spent medium is able to induce dormant *M. smegmatis* cells to grow, confirming that the growth induction component of the scout model is likely applicable to this species. This is the principle result of the concluding part of my investigation.

Elucidation of the chemical structure of compound(s) composing Fraction 17 is beyond the scope of my project, and will be addressed in future studies. However, it was tempting to conduct a few additional, unplanned experiments, which may be helpful in the future studies of growth induction in *M. smegmatis*. Chemical analysis revealed that the major compound present in the FT Fraction was C₈H₁₂N. One – admittedly speculative – possibility is that the compound in question is phenylethylamine. We applied 2-phenylethylamine hydrochloride, in various concentrations, to suspensions of dormant cells of *M. smegmatis*, and observed an induction of growth (Figures 5.8-9). The pattern of concentration dependency of this induction activity is not clear, and a larger scale bioassay is underway.
Section 6. Conclusions.

The preceding experiments confirmed the major predictions of the scout model for taxonomically diverse set of species.

The behavior of marine bacteria in a long-term incubation supported the idea of dormancy as a major strategy employed by environmental species. The varied time points at which the same species displayed visible growth, even though the conditions of incubation were not changing, indicates that their awakening was random, and not a response to environmental cues. Further, the growth resumption of dormant cells of *M. smegmatis* and *E. coli*, also followed a stochastic pattern of awakening. The latter suggests that the time of awakening may be governed by processes within the cell, rather than in its surrounding.

A second prediction of the scout model, that actively growing kin cells can induce non-growing cells to grow, was confirmed for dormant cells of *M. smegmatis*. While my collaborators and I have not yet been able to elucidate the chemical structure of the activity involved, it is clear that it is not the resuscitation-promoting factor RPF (Mukamolova et al. 1998) and is thus likely novel for biology of *M. smegmatis*. The study of this inducing activity is in progress. This line of research is important since *M. smegmatis* is a model organism for studying tuberculosis.
Collectively these data provide a strong support for the existence of a microbial survival strategy, in both the environment and in the human body, that is based on stochastic exit from dormancy by scout cells, and signaling the success of the scout to the rest of the (still dormant) population. This has important implications for further study.

In environmental microbiology, the scout model represents a partial explanation for the ‘Great Plate Count Anomaly,’ (Staley and Konopka 1985), and opens up possibilities to develop better tools for the recovery novel species from the large reservoir of currently uncultivated species. Access to these species is both a matter of great interest for ecological studies and for the potential for isolation of new chemical entities that could be utilized in the treatment of diseases, development of biofuels, and other potential applications.

The implications for pathogenic organisms are equally significant. As latent infections are notoriously difficult to treat, understanding the state of the cells during latency is of paramount importance. For pathogenic microorganisms that conform to the scout model, new treatments could be developed to target essential processes that occur even under dormant conditions. Perhaps even more effective, wakening up dormant pathogens along with currently available treatments known to eliminate actively growing cells could be a useful way to treat infections.

In short, this study makes it likely that microbial life cycle consists of periods of dormancy, and periods of activity, with stochastic processes governing the transition
from the former to the latter. Further studies of these processes may have implications in many disciplines, from environmental microbiology to disease control.
Appendix. Supplementary Material from Section 2.

Figure A.1 Prototypes of Teflon Chips.
Figure A.2 Teflon Chip.
Figure A.3. Cells from a well exhibiting growth after 4 months.

Figure A.4. Cells from a well exhibiting growth after 8 months.
Figure A.5. Cells from a well exhibiting growth after 11 months.
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Figure A.6 Full list of species, identified by the percent similarity of their 16s rRNA gene sequence to the closest relative reported in GenBnk). Time point at which visible growth was noted is indicated by blue boxes. Numbers in blue boxes indicate the number of isolates that appeared at the given time point.
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**Figure A.7** Percent recovery of cells of selected species during single-cell based subculturing of biomass obtained in long-term incubation of an environmental sample.
References:


Oliver JD (2005) The Viable but Nonculturable State in Bacteria. *The Journal of Microbiology* 43(S): 93-100.1


