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COMPARATIVE ECOLOGICAL ANALYSIS OF RIBOSOMAL RNA GENE COPY NUMBER IN HETEROTROPHIC SOIL BACTERIA

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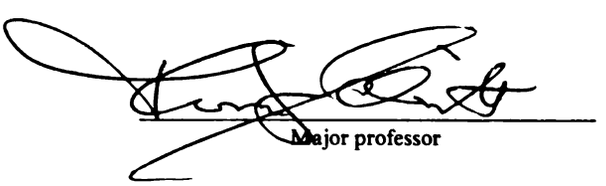

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# COMPARATIVE ECOLOGICAL ANALYSIS OF RIBOSOMAL RNA GENE COPY NUMBER IN HETEROTROPHIC SOIL BACTERIA

Joel Albert Klappenbach

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology & Molecular Genetics

2001

# ABSTRACT

## COMPARATIVE ECOLOGICAL ANALYSIS OF RIBOSOMAL RNA GENE COPY NUMBER IN HETEROTROPHIC SOIL BACTERIA

By

Joel Albert Klappenbach

Ribosomal RNA genes are commonly present in many copies in a bacterial genome among a background of primarily single copy genes. The number of rRNA operons in a genome is a phylogenetically distributed trait within the domain *Bacteria*, though conserved within individual taxonomic groups. The synthesis of ribosomes is the single largest metabolic expenditure of a bacterial cell, and ribosomal RNA gene copy number imposes a limit on the rate of ribosome biosynthesis. A comparative approach was used in this dissertation to investigate the ecological implications of rRNA gene copy number in heterotrophic soil microbial communities. Bacteria with the same number of rRNA genes were found in divergent phylogenetic lineages and a correlation between evolutionary ancestry and rRNA gene copy number was apparent between strains, species, genera, and higher taxonomic levels. A correlation between evolutionary ancestry and rRNA gene copy number among distantly related bacteria sharing similar ecological niches indicated that rRNA copy number is influenced by environmental selective pressures. Soil microbial communities provided a model system to investigate the relationship between rRNA gene copy number and bacterial response to increased nutrient availability.

rRNA gene copy number correlated with the time required for phylogenetically diverse bacteria to form colonies on solid agar media, indicating phenotypic effects associated with rRNA gene copy number. Soil bacteria that rapidly formed colonies possessed a significantly greater number of rRNA genes per genome than later appearing colonies. The hypothesis was tested that heterotrophic soil bacteria with many rRNA genes possess an increased capacity for rRNA synthesis permitting rapid increases in cellular rRNA content and growth. In soil microcosms amended with succinate, rapid increases in rRNA abundance and growth were observed in phylogenetic groups of bacteria possessing many (≥4) rRNA genes per genome. In contrast, increases in rRNA abundance and growth were not observed in phylogenetic groups of bacteria with few (≤3) rRNA genes following succinate amendment. Bacteria with few rRNA genes per genome comprised the largest fraction of the soil microbial community. An ecological trade-off in growth rate versus numerical growth yield was rejected as an explanation for the high abundance of slow growing bacteria in soil. Maximal growth rates were positively correlated with rRNA gene copy number, however, several bacteria with many rRNA operons were unable to grow at low nutrient concentrations. The research described in this dissertation demonstrates that rRNA gene copy number reflects the metabolic capacity of bacteria for rRNA synthesis and growth, providing a genetic indicator of bacterial ecological strategies for responding to nutrient availability.

# ACKNOWLEDGEMENTS

I am indebted to many individuals who provided me with support and guidance during the past six years. Thomas Schmidt, my advisor, afforded me the creative freedom and intellectual guidance to develop into the scientist and scholar that I am today. His example as a mentor, teacher, and scholar will continue to provide direction throughout my career. My fellow students and friends in the Schmidt and Breznak labs created an environment that continually challenged my scientific thinking and helped me develop the confidence I possess as a scientist. Although we often made light of our habitual lunch hour and following coffee breaks as a group, it was the scientific discussions and humor relayed during these times such as these that made us both good friends and confident scholars. I will miss these times. I began this journey with my wife Becky over five years ago, a long way from our home and families in Seattle. The love, patience, and support of my wife Becky and our parents and families made this arduous journey a wonderful experience I will never forget. Thank you all.

# PREFACE

The work presented in this dissertation could not have been completed without the assistance of others. I would like to recognize the intellectual contributions and computational assistance of Dr. James R. Cole and Paul R. Saxman in the creation of the rrndb, the Ribosomal RNA Operon Copy Number Database, an Internet-accessible database presented in Chapter 2 and in: Klappenbach, J.A., P.R. Saxman, J.R. Cole, T.M. Schmidt (2001) rrndb: the Ribosomal RNA operon copy number database. Nucl. Acids. Res. 29:181-184. The relationship I established between bacterial response time and rRNA operon copy number in Chapter 3 provided an intellectual foundation for the formal presentation of data from the dissertation of Dr. John M. Dunbar, which is presented in Figure 4.4 of this work and in: Klappenbach, J.A., J.M. Dunbar, T.M. Schmidt. (2000) rRNA operon copy number reflects ecological strategies of bacteria. Appl. Env. Microbiol. 66:1328-1333.

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# CHAPTER 1

## RIBOSOMAL RNA GENE REDUNDANCY IN PROKARYOTES

### INTRODUCTION

Prokaryotic genomes are principally composed of unique copies of protein-encoding genes. Despite apparent evolutionary selection against gene redundancy in prokaryotic genomes, the genes encoding the structural rRNA molecules are commonly observed in many copies (58). Unlike expression of the ribosomal protein-encoding genes, the rate of rRNA synthesis cannot be increased through translation amplification – rRNA transcripts are the functional products (Figure 1.1). Using the length of an rRNA operon, the transcriptional rate of RNA polymerase, and the maximum density of RNA polymerase molecules per gene, theoretical rates of ribosome synthesis can be calculated. These calculations indicate that a single rRNA operon is insufficient to supply the number of ribosomes required to achieve maximal growth rates observed in *E. coli* (8). Gene redundancy is the primary mechanism for increasing the rate of ribosome synthesis above limits imposed by rRNA transcription. Rates of ribosome synthesis increase with growth rate to support an increased demand for protein synthesis. During rapid growth, transcription of the rRNA genes can account for up to 70% of total cellular transcription (9). Variation in rRNA gene copy number among a background of single-copy genes and the central physiological importance of the ribosome suggest that rRNA gene redundancy is of evolutionary adaptive significance.

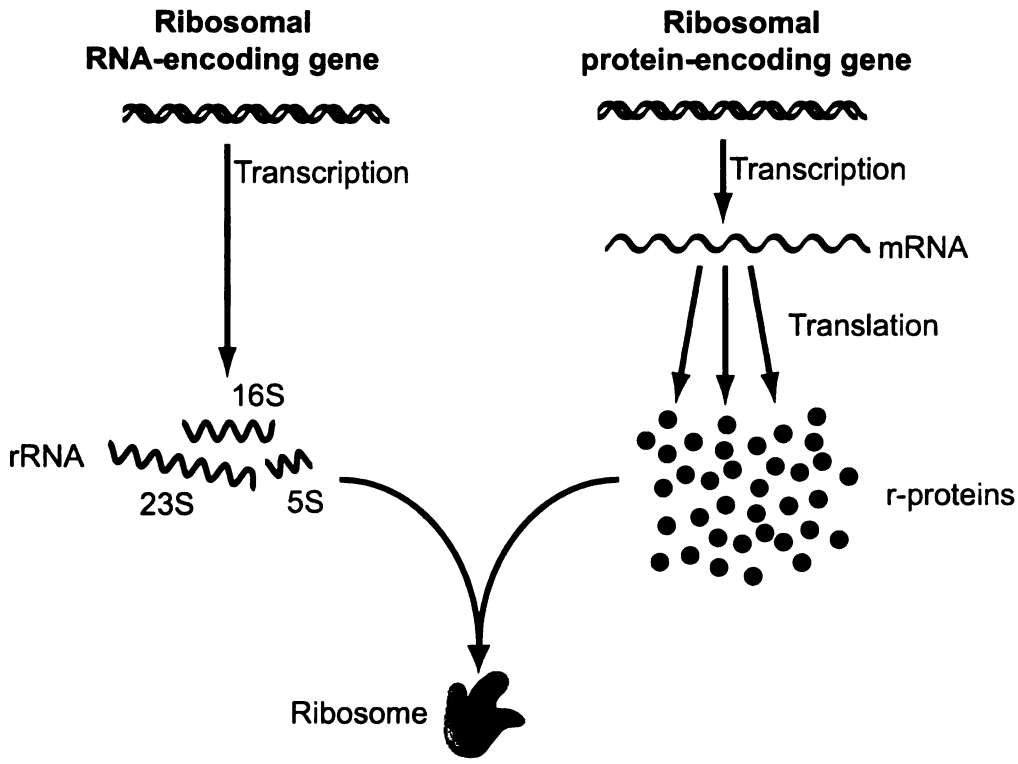


**FIGURE 1.1.** Synthesis and assembly of the ribosome.

The focus of this dissertation is to determine whether rRNA gene redundancy is an adaptively significant trait indicating the general ecological strategies of heterotrophic soil bacterial populations. In this chapter, I discuss rRNA gene organization, transcriptional regulation, and the role of rRNA gene redundancy in cellular metabolism. The phylogenetic distribution of rRNA gene copy number in the ecological context of bacterial populations and soil microbial communities are also presented. Lastly, I provide an outline of this dissertation and the specific questions I sought to address through my research. The comparative analysis of a biological trait across taxonomic boundaries requires an understanding of both the physiological consequences and the phylogenetic distribution of the trait under consideration (17, 24). Understanding genetic determinants of microbial competitiveness is critical to our knowledge of factors influencing the diversity and structure of extant microbial communities.

## BACKGROUND

### Organization and Location of rRNA Genes

Clustering of rRNA genes into operons is common in bacterial genomes and results in transcription of equimolar quantities of each rRNA gene (Figure 1.2). However, alternative organizations of rRNA genes are observed in some bacteria indicating that organization into operons is not a strict requirement (6, 53, 59, 68). Genes encoding transfer RNAs (tRNAs) are often located in the internally transcribed spacer (ITS) region and distal to the 5S rRNA gene (23). The ITS region is variable in length and contributes to the majority of sequence

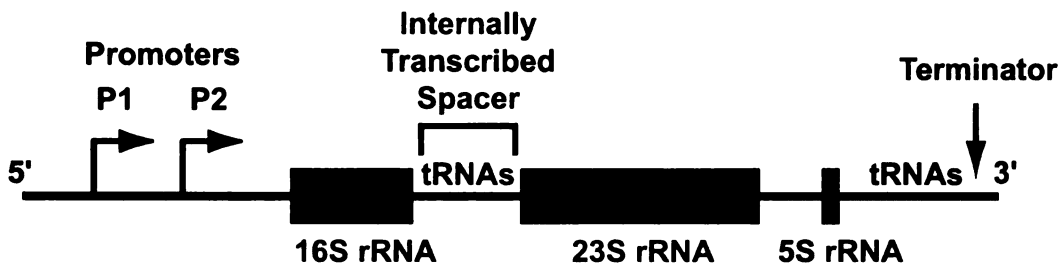


**FIGURE 1.2.** Typical organization of a rRNA operon.

diversity among many rRNA operons in many species of bacteria (5, 31, 44). Introns in rRNA coding regions have also been documented in several archaeal species (29, 33, 47). Organization of rRNA operons around the origin of replication is a common structural feature among phylogenetically diverse bacteria. Localization of rRNA operons near the origin of replication provides a gene dosage effect during rapid growth where the effective number of rRNA operons in *Escherichia coli* can be as high as 36 copies during exponential growth ($\mu = 2.5\ h^{-1}$) due to multiple replication forks (9).

**Regulation of rRNA synthesis**

Our understanding of rRNA transcriptional regulation is primarily derived from studies of *E. coli*. Transcription of all seven rRNA operons in *E. coli* is coordinately regulated by two tandem promoters, P1 and P2, roughly 100 bp upstream of the transcriptional start site (Figure 1.2). Initiation of transcription occurs predominantly at the P2 site following an upshift in precursor pools, and shifts to P1 initiation once steady state growth rates are achieved (20). The P2

promoter is thought to be a low level constitutive promoter at all steady-state growth rates (56), and therefore responsible for "excess" rRNA (see discussion below) observed during slow growth (23). Differential transcription initiation has also been observed in *Mycobacterium* spp., which can possess up to five promoters controlling a single rRNA operon (21). Two models of rRNA transcription control are widely supported: growth rate-dependent and stringent control.

Stringent control describes the response of a cell to amino acid starvation. When a cell is starved for an amino acid, it responds by rapidly shutting down synthesis of stable RNA (rRNA and tRNA) (11). The shutdown of stable RNA synthesis is accompanied by an intracellular increase of guanosine tetra- and penta-phosphate (ppGpp) (18). The ppGpp molecule is thought to physically interact with RNA polymerase to prevent transcriptional initiation, thereby rapidly shutting down stable RNA synthesis (4, 70). The stringent response is observed in different species of bacteria, suggesting similar regulatory mechanisms for ribosome biosynthesis (61, 66).

The growth rate-dependent model of transcriptional control is based on the positive correlation observed between growth rate and the cellular concentration of stable RNA (57). A proposed model of homeostatic control in *E. coli* directly relates ribosome synthesis to resource availability for growth (20). Resources available for biosynthesis and growth determine the intracellular concentrations of GTP and ATP, which stabilize open initiation complexes at rRNA promoters. Stable initiation complexes lead to full-length rRNA transcripts

and functional ribosomes. Ribosomal RNA molecules regulate transcription of ribosomal proteins to ensure that equimolar quantities of each component are available for ribosome assembly (32). These control mechanisms also provide feedback mechanisms to down-regulate ribosome biosynthesis when intracellular pools of GTP and ATP decline as resources are exhausted. The growth rate-dependent model of control is supported by experimental observations that the functional inactivation of rRNA operons in *E. coli* causes increased expression from the remaining intact copies (12).

**Variation of Ribosomal RNA Operon Copy Number in the *Bacteria***

The number of rRNA operons among prokaryotic microorganisms varies from one to fifteen copies per chromosome (37, 52). Bacteria sharing evolutionary ancestry and ecological niches often possess the same number of rRNA operons per genome. The pathogenic bacteria *Rickettsia prowazkeii* (2) and *Mycoplasma pneumoniae* (7) have one rRNA operon, while the enteric bacteria *E. coli* (16) and *Salmonella typhimurium* (1) each possess seven copies per genome. The highest known number of rRNA operons per genome can be found among spore-forming bacteria isolated from soil; *Bacillus subtilis* (40) and *Clostridium paradoxum* (52) possess 10 and 15 copies, respectively. Shared life-histories among bacteria with similar numbers of rRNA operons per genome suggests an evolutionary link between ecological strategies and rRNA operon copy number.

If rRNA operon multiplicity was exclusively determined by ancestry, a strict correlation between phylogeny and rRNA operon copy number would be

expected. However, phylogenetic analysis of 16S rRNA sequence data suggests that phylogenetic relatedness is not the sole determinant of rRNA operon copy number (Figure 1.3). Bacteria with identical rRNA operon copy numbers appear to have evolved convergently in several phylogenetic lineages. The probability of bacteria possessing the same number of rRNA operons increases with decreasing phylogenetic distance. For example, species in the $\alpha$-class of the *Proteobacteria* phylum possess between one and three rRNA operons, while species in the $\gamma$–class of the *Proteobacteria* phylum typically possess between five and six rRNA operons per genome. Correlations between phylogeny and rRNA operon copy number are also apparent at the genus level within commonly recognized bacterial groups. The *Actinobacteria* phylum (Gram positive bacteria with high mol %G+C genomes) contains the *Mycobacterium* genus with species possessing 1 to 2 rRNA operons per genome, and *Streptomyces* genus with species possessing 4 to 7 rRNA operons per genome. Convergent evolution of rRNA operon copy number in distantly related bacteria sharing similar life-histories indicates that the number of rRNA operons per genome is a biological trait of potential adaptive significance. Selective pressures influencing rRNA operon copy number in bacteria sharing similar ecological niches describes the role in which rRNA operon copy number may be adaptively significant.

**FIGURE 1.3. (A)** *Bacteria.* **(B)** *Bacteria and Archaea.* rRNA operon copy number mapped onto organismal phylogeny. Phylogenetic sub-trees extracted from the RDP (41). Branch lengths do not necessarily represent the actual evolutionary distances, but topology is preserved. Values to the right of species' names indicate the number of rRNA operon equivalents per genome. rRNA operon copy numbers were obtained from the literature and do not necessarily correspond to the sequence of the strain designation used for phylogenetic reconstruction (34). Major phylogenetic groups listed on right. Abbreviations are as follows: C/F/B, Cytophaga/Flexibacter/Bacteroides; Cyano, *Cyanobacteria*; Pla, *Planctomycetes*; Therm, thermophiles.

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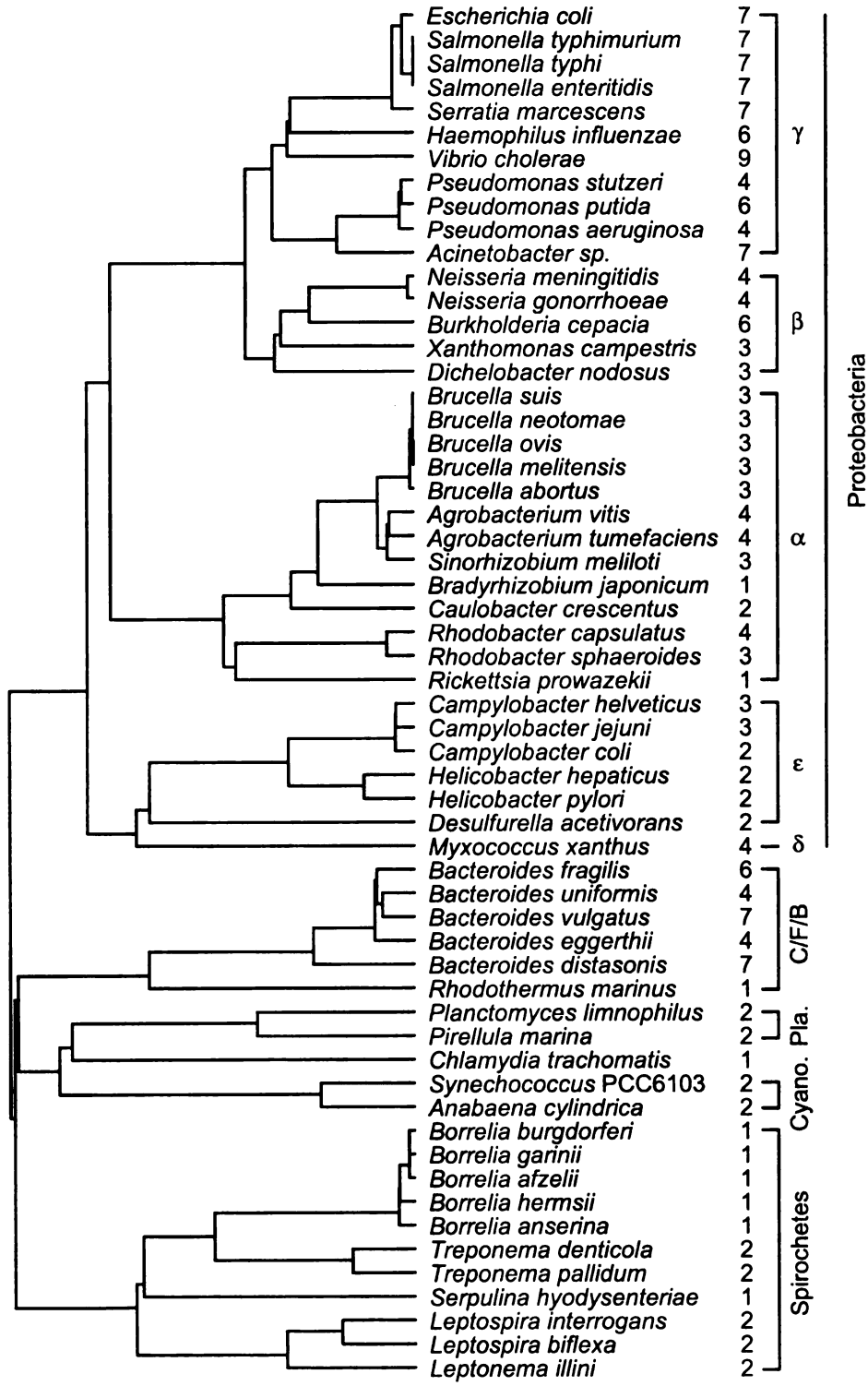
Escherichia coli 7
Salmonella typhimurium 7
Salmonella typhi 7
Salmonella enteritidis 7
Serratia marcescens 7
Haemophilus influenzae 6
Vibrio cholerae 9
Pseudomonas stutzeri 4
Pseudomonas putida 6
Pseudomonas aeruginosa 4
Acinetobacter sp. 7
Neisseria meningitidis 4
Neisseria gonorrhoeae 4
Burkholderia cepacia 6
Xanthomonas campestris 3
Dichelobacter nodosus 3
Brucella suis 3
Brucella neotomae 3
Brucella ovis 3
Brucella melitensis 3
Brucella abortus 3
Agrobacterium vitis 4
Agrobacterium tumefaciens 4
Sinorhizobium meliloti 3
Bradyrhizobium japonicum 1
Caulobacter crescentus 2
Rhodobacter capsulatus 4
Rhodobacter sphaeroides 3
Rickettsia prowazekii 1
Campylobacter helveticus 3
Campylobacter jejuni 3
Campylobacter coli 2
Helicobacter hepaticus 2
Helicobacter pylori 2
Desulfurella acetivorans 2
Myxococcus xanthus 4
Bacteroides fragilis 6
Bacteroides uniformis 4
Bacteroides vulgatus 7
Bacteroides eggerthii 4
Bacteroides distasonis 7
Rhodothermus marinus 1
Planctomyces limnophilus 2
Pirellula marina 2
Chlamydia trachomatis 1
Synechococcus PCC6103 2
Anabaena cylindrica 2
Borrelia burgdorferi 1
Borrelia garinii 1
Borrelia afzelii 1
Borrelia hermsii 1
Borrelia anserina 1
Treponema denticola 2
Treponema pallidum 2
Serpulina hyodysenteriae 1
Leptospira interrogans 2
Leptospira biflexa 2
Leptonema illini 2
γ
β
α
ε
δ
Proteobacteria
C/F/B
Pla.
Cyano.
Spirochetes

**FIGURE 1.3, A**

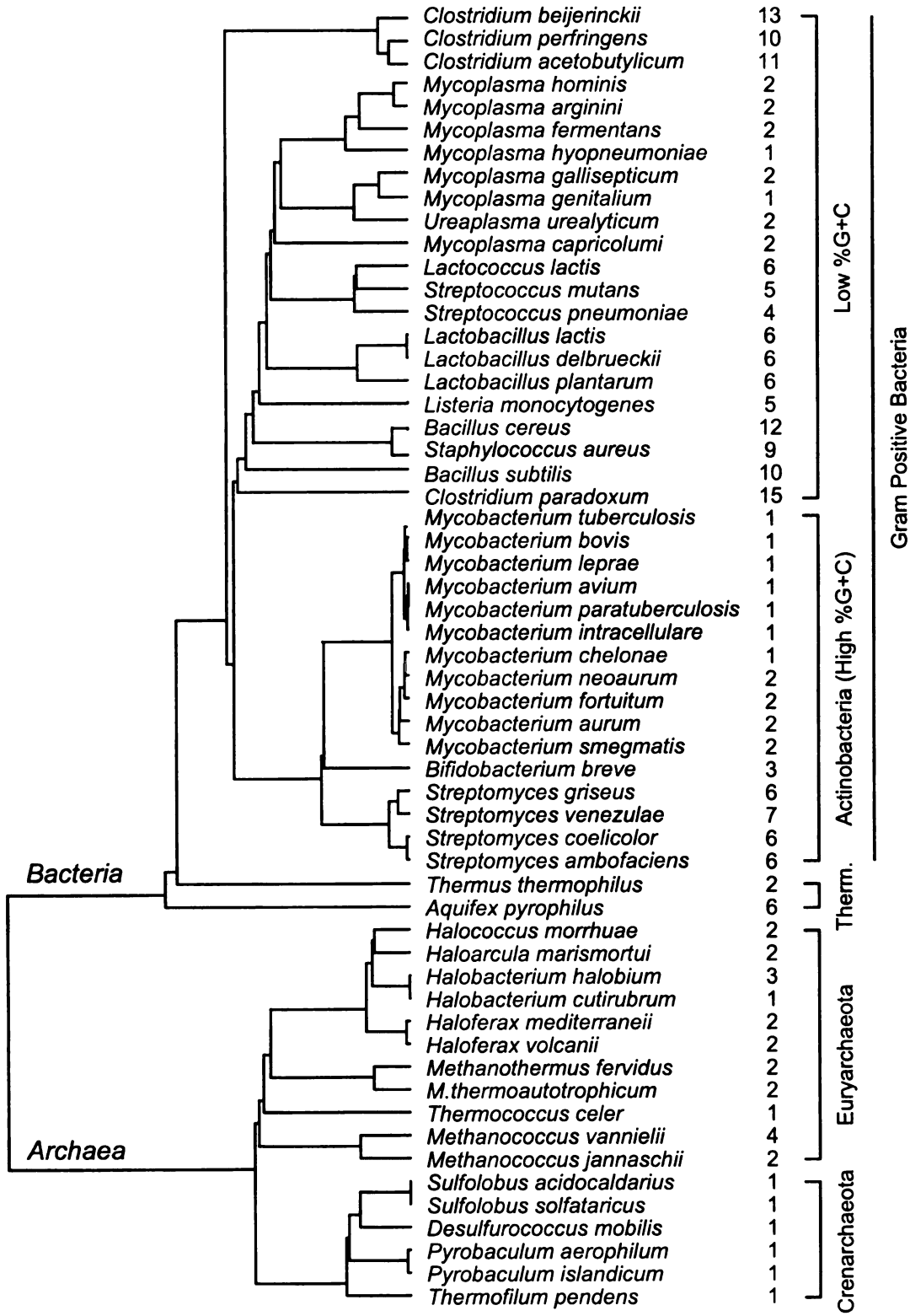
Clostridium beijerinckii 13
Clostridium perfringens 10
Clostridium acetobutylicum 11
Mycoplasma hominis 2
Mycoplasma arginini 2
Mycoplasma fermentans 2
Mycoplasma hyopneumoniae 1
Mycoplasma gallisepticum 2
Mycoplasma genitalium 1
Ureaplasma urealyticum 2
Mycoplasma capricolumi 2
Lactococcus lactis 6
Streptococcus mutans 5
Streptococcus pneumoniae 4
Lactobacillus lactis 6
Lactobacillus delbrueckii 6
Lactobacillus plantarum 6
Listeria monocytogenes 5
Bacillus cereus 12
Staphylococcus aureus 9
Bacillus subtilis 10
Clostridium paradoxum 15
Mycobacterium tuberculosis 1
Mycobacterium bovis 1
Mycobacterium leprae 1
Mycobacterium avium 1
Mycobacterium paratuberculosis 1
Mycobacterium intracellulare 1
Mycobacterium chelonae 1
Mycobacterium neoaurum 2
Mycobacterium fortuitum 2
Mycobacterium aurum 2
Mycobacterium smegmatis 2
Bifidobacterium breve 3
Streptomyces griseus 6
Streptomyces venezulae 7
Streptomyces coelicolor 6
Streptomyces ambofaciens 6
Thermus thermophilus 2
Aquifex pyrophilus 6
Halococcus morrhuae 2
Haloarcula marismortui 2
Halobacterium halobium 3
Halobacterium cutirubrum 1
Haloferax mediterraneii 2
Haloferax volcanii 2
Methanothermus fervidus 2
M.thermoautotrophicum 2
Thermococcus celer 1
Methanococcus vannielii 4
Methanococcus jannaschii 2
Sulfolobus acidocaldarius 1
Sulfolobus solfataricus 1
Desulfurococcus mobilis 1
Pyrobaculum aerophilum 1
Pyrobaculum islandicum 1
Thermofilum pendens 1
Bacteria
Archaea
Low %G+C
Actinobacteria (High %G+C)
Therm.
Euryarchaeota
Crenarchaeota
Gram Positive Bacteria

**FIGURE 1.3, B**

## Consequences of rRNA Gene Redundancy in Prokaryotes

### *Sequence Conservation*

The catalytic and structural functions of rRNA impose strong selective pressures on the conservation of primary nucleotide sequence (45, 46, 67). The conservation of ribosome function is so strong that rRNA genes can be experimentally interchanged between taxonomic domains (*Eukarya* and *Bacteria*) to provide the sole source of rRNA for protein synthesis (3). Whether horizontal transfer of rRNA genes occurs in nature is the subject of much debate (43, 69, 71, 72). The coding regions of the rRNA genes are often identical within a genome (Table 1.1), yet in a few instances intragenomic rRNA sequence variability as high as 10% has been documented (72). The observation that intragenomic rRNA sequence variability exceeds intergenomic variability between closely related bacteria suggests that rRNA gene duplication and divergence is the primary mechanism for sequence variability within rRNA genes in a single genome (38, 48). High rates of intragenomic gene conversion between experimentally mutated and wild-type rRNA operons in *E. coli* demonstrate strong selective pressure for the maintenance of extant rRNA sequences within a bacterial genome (26).

# TABLE 1.1. Intragenomic variability in rRNA coding regions[a].

| Organism[b] | No. rRNA operons | 16S diff (nt) | % diff (16S) | 23S diff (nt) | % diff (23S) |
|---|---|---|---|---|---|
| *Aquifex aeolicus* VF5 | 2 | - | - | - | - |
| *Bacillus subtilis* ATCC 23857 | 10 | 1-15 | 0.97 | 1-35 | 1.19 |
| *Campylobacter jejuni* ATCC 700819 | 3 | - | - | - | - |
| *Escherichia coli* ATCC 10798 | 7 | 0-19 | 1.23 | 0-23 | 0.79 |
| *Haemophilus influenzae* ATCC 51907 | 6 | - | - | - | - |
| *Helicobacter pylori* 26695 | 2 | - | - | 4 | 0.13 |
| *Methanococcus jannaschii* DSMZ 2661 | 2 | 3 | 0.2 | 9 | 0.31 |
| *M.thermoautotrophicum* ATCC 29096 | 2 | 2 | 0.14 | 15 | 0.49 |
| *Neisseria menigitidis* MC 58 | 4 | - | - | - | - |
| *Treponema pallidum* ATCC 25870 | 2 | - | - | - | - |
| *Ureaplasma urealyticum* serovar 3 | 2 | 1 | 0.07 | 4 | 0.14 |
| *Vibrio cholerae* ATCC 39315 | 8 | 0-14 | 0.91 | 0-29 | 1 |
| *Xyella fastidosa* 9a5c | 2 | - | - | - | - |

[a] data adapted from Chapter 2 & ref. 29
[b] data from full genome sequencing projects
diff, differences
-, no differences

rRNA gene redundancy provides regions of highly conserved sequence that are involved in intragenomic recombination events. Inversions can occur between rRNA operons, but are generally unstable and have deleterious effects on growth rate (25, 27, 28, 65). Gene duplication has been proposed as an adaptive mechanism whereby cells may increase the dosage of particular genes under nutrient limitation (62), but duplication of rRNA genes has only been documented in eukaryotic organisms (39, 54). Homologous recombination between multiple rRNA operons may increase the relative fitness of a bacterium by removing deleterious mutations in rRNA genes. Resistance to ribosome-targeting antibiotics also imposes selective pressure for the maintenance of multiple rRNA operons (50).

### *Specific Function*

Reporter gene fusion studies of rRNA operon promoters in *E. coli* indicate that no single operon is uniquely regulated during a particular mode of growth (14). Transcription of the seven operons in *E. coli* is coordinated such that all operons are transcribed over a wide range of growth rates supported by different media compositions (14). The transcribed rRNA species from each of the seven rRNA operons in *E. coli*, although not identical in sequence, seem to be functionally equivalent. The metabolically versatile *Rhodobacter sphaeroides* possesses three rRNA operons that are all equivalently transcribed during photosynthetic, aerobic, and anaerobic modes of growth (15).

The nucleotide composition of rRNA molecules can affect the performance of ribosomes under different physical conditions. Psychotolerant nucleotide

signatures in *Bacillus cereus* rRNA genes correlate with increased growth rates at low temperatures (51). Similarly, increased mol%G+C base compositions are associated with thermostability of rRNA secondary structures at high temperatures (10). While rRNA genes are differentially transcribed in some species and differ in translational performance under certain conditions, ribosomes are thought to possess equivalent functionality among bacteria.

***Steady-state growth rate effects***

rRNA gene copy number imposes a theoretical limit on the maximal growth rate attainable by bacterial cells. Thus, it is generally perceived that multiple rRNA operons are required to achieve high growth rates. However, inactivation or deletion of rRNA operons has little effect on maximal growth rates in bacteria with multiple rRNA operons (13, 49, 63). *Mycobacteria* spp. with a single rRNA operon can grow at rates equivalent to species with two rRNA operons by increasing translational efficiency using multiple (3 – 5) promoters (22). In addition, the extreme thermophile *Thermococcus celer* can reach a doubling time of less than 50 minutes with a single rRNA operon, much faster than several mesophilic bacteria with many rRNA operon copies (73). Therefore, a simple relationship between rRNA operon copy number and growth rate is not immediately evident.

Constitutive expression of multiple rRNA operons may confer a metabolic burden on slowly growing cells due to the production of superfluous ribosomes. Extra copies of plasmid-borne rRNA operons increase stable RNA concentrations while concomitantly decreasing growth rates on minimal media in

*E. coli* (64); this may indicate a cost associated with constitutive expression from multiple rRNA operons. On nutritionally rich medium supporting high maximal growth rates, extra rRNA operons seem to have little effect on growth rate or stable RNA concentrations (64). It is unknown whether rRNA is constitutively expressed at low growth rates in bacterial species other than *E. coli.*

***Transitional growth rate effects***

Perhaps the most significant demonstration of the physiological effects associated with rRNA redundancy is the correlation between rRNA operon copy number and shift-up time in *E. coli* (13). Functional inactivation of rRNA operons in *E. coli* increases the time required to shift from low to high growth rates when cells are transferred from minimal to rich medium or from 25°C to 42°C (13). As copies of the rRNA operon are sequentially inactivated, the shift-up time concomitantly increases. Condon et al. (13) suggested that selective pressures on the ability to rapidly increase growth rates may explain the presence of multiple rRNA operons in *E. coli.* Accordingly, bacteria such as *Bacillus* and *Clostridium* spp. that sporulate during starvation typically possess ≥10 rRNA operons per genome (Figure 1.3). Spore forming bacteria must rapidly germinate from a resting spore in order to compete during conditions favoring growth. rRNA gene redundancy provides multiple sites to initiate transcription, permitting rapid increases in the protein synthesis machinery necessary for growth.

Constitutive expression of rRNA genes during starvation may also permit rapid response rates to nutrient availability. Upon nutrient shift-up in *E. coli*,

protein synthesis rates are initially faster than can be explained by the synthesis of new ribosomes (36). Koch (1971) has proposed that the maintenance of ribosomes in "excess" of translational demand at low growth rates is an adaptation of *E. coli* to the "feast-or-famine" environment of the mammalian digestive system (35). In an environment such as the intestinal lumen, it would be advantageous for *E. coli* to be able to rapidly initiate cell division in response to transient fluctuations in nutrient availability. Advantages associated with maintenance of inactive ribosome pools during starvation would only be realized during frequent nutrient fluctuations, due to the fitness costs associated with transcription of rRNA during slow growth or prolonged starvation.

## THESIS OUTLINE

All organisms allocate limited resources to the competing demands of growth, maintenance, and reproduction. Variations in the distribution and abundance of resources are important selective pressures influencing microbial populations. This dissertation applies a comparative approach to elucidate the ecological significance of rRNA operon copy number in heterotrophic bacterial populations from soil. It was postulated that:

> *Ribosomal rRNA operon copy number is an adaptively significant trait indicating the general ecological strategies of heterotrophic soil bacteria for responding to nutrient availability.*

Soil is an environment rich in microbial diversity that can vary considerably with regard to resource availability. Resources are made available in soils through precipitation, agricultural fertilization, biotic and abiotic perturbations, and rhizodeposition by overlying plant communities (19, 30, 55, 60). The temporal frequency of these events can vary on diurnal, seasonal, and historical scales. Fluctuations in resource availability could influence the population structure of communities in soil with respect to rRNA operon copy number, alternately favoring the abundance of populations with many or few rRNA operons. Thus, bacterial populations possessing both many and few rRNA operons are expected to coexist as communities in soil.

An adaptation is defined as a phenotype reflecting underlying genetic change, permitting the growth and persistence of an organism in a particular

biological niche. Identifying a biological trait as adaptively significant among a collection of organisms requires multiple independent phylogenetic observations (17). Additionally, apparent life-history traits are often the result of phenotypic plasticity and not underlying genetic change (42). Therefore, phylogenetic relationships are a critical component of the comparative analysis of biological traits among organisms. In Chapter 2, a phylogenetically arranged database of organisms with known rRNA operon copy numbers was assembled in order to map the relationship between rRNA gene redundancy and organismal ancestry. Information obtained from the literature and full-genome sequencing projects was comparatively analyzed at phylogenetic levels including strain, species, genus, and higher taxonomic levels. Correlations between phylogeny and rRNA operon copy number were used to select rRNA-based probes to monitor the response of specific bacterial populations in soil (Chapter 4).

The hypothesis that bacteria with many (≥4) rRNA operons respond to nutrient availability more rapidly than bacteria with few (≤3) rRNA operons was tested in Chapter 3. A bacterial community from soil was characterized based on the time required for individual populations to form visible colonies on solid agar media. Colonies were selected from early and late time intervals and characterized by rRNA operon copy number and 16S rRNA-based phylogeny. Transcription of rRNA is regulated by resource availability. Bacteria with multiple rRNA operons possess multiple sites to initiate and sustain transcription, allowing for rapid growth. The observation that some bacteria with multiple rRNA operons maintain excess translation capacity during starvation also indicates that these

bacteria are capable of rapidly initiating growth in response to nutrient amendment.

In Chapter 4, the correlation between rRNA operon copy number, phylogeny (Chapter 2), and cultivation time (Chapter 3) was used to predict the response of soil bacteria to succinate amendment in soil microcosms. The responses of soil bacteria were assessed by: 1) the time required to initiate rRNA synthesis following amendment, 2) the magnitude of change in rRNA synthesis following amendment, and 3) the time required for colony formation after plating. Soil microcosms were constructed from soils obtained at the Kellogg Biological Station Long-Term Ecological Research site. A destructive sampling scheme was employed in which triplicate microcosms were removed at each time point for each treatment. Treatments included an aqueous succinate solution (1 mg C•g $soil^{-1}$) or water alone, both of which were wetted to 40% of field water holding capacity. Microcosms receiving treatment were compared to unamended microcosms that were homogenized at the time of treatment. A combination of direct (colony response curves, direct counts) and indirect (rRNA hybridization) methods provided a means to relate changes in rRNA synthesis with changes in the physical structure of the bacterial community.

Lastly, in Chapter 5, an ecological trade-off between maximum growth rate and numerical cell yield was tested as a potential mechanism explaining the high abundance of slowly growing bacteria in soil. A collection of soil bacteria, isolated based on colony formation time (Chapter 4), were grown on a nutritionally complex medium over a 5000-fold concentration range. Numerical

cell yields (cells per mL of medium) were compared at five different media concentrations, and growth rates were determined under substrate saturating conditions. Results were analyzed using both traditional parametric statistics and phylogenetically independent contrasts to control for non-independence of ancestrally related organisms.

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# CHAPTER 2

## A PHYLOGENETICALLY STRUCTURED DATABASE FOR RIBOSOMAL RNA OPERON COPY NUMBER IN BACTERIAL GENOMES

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## INTRODUCTION

Microbes are the most abundant and most diverse form of life on earth (17). Despite their ubiquity, it is clear from gene sequence studies that only a small percentage of microbes (0.1 – 0.5 %) have been cultivated in the laboratory (15). Identification and classification of microbes is further confounded by a general absence of morphologically distinct features – thousands of bacterial species can be categorized by a few different (ca. 17) morphologies. For the past 10 – 15 years, microbiologists have relied upon DNA sequence information for microbial identification, based primarily on the genes encoding the small subunit RNA molecule of the ribosome (16S rRNA or SSU rRNA). Functional constraints on the translational apparatus limit variability in the 16S rRNA molecule, resulting in a high degree of sequence conservation. The conservation of the rRNA gene sequence permits bacterial characterization based on sequence information obtained from pure cultures or cloned genes from mixed communities. A priori knowledge of rRNA sequence data can be used to design phylogenetically conserved probes that target both individual and closely related groups of microorganisms without cultivation. A principle repository of 16S rRNA sequences, the Ribosomal Database Project, currently maintains over 17,000 aligned entries (12425 sequences ≥ 900 bp) representing 850 of 940 formally recognized prokaryotic genera, which are placed into 1149 phylogenetic groups (12).

The ribosomal RNA genes (16S, 23S & 5S subunits) are typically linked together with tRNA molecules into operons that are coordinately transcribed to produce equimolar quantities of each gene product. During rapid exponential growth ($\mu = 2.5\ h^{-1}$), the effective number of rRNA operons in *Escherichia coli* can be as high as 36 copies (1). Sequence heterogeneity exists among multiple rRNA genes encoded on a single genome, yet little evidence exists suggesting functional independence (4, 6). While reports of intra-genomic variability of 16S rRNA range as high as 6.5% (16), an analysis of complete genome sequences stored in the *rrndb* indicates a maximum of 1.23% (*E. coli* ) among the 14 species examined (Table 2.1). Both rRNA operon redundancy and intra-genomic sequence heterogeneity have important practical implications for researchers attempting to identify and quantify bacteria using rRNA sequence data (7, 18).

Molecular methods for microbial diversity assessment rely primarily on PCR-amplification of 16S rRNA genes from complex samples followed by 1) cloning and sequencing of unique amplicons, 2) separation of amplicons based on chemical composition via denaturing- or temperature-gradient gel electrophoresis (13, 14), or 3) separation of amplicons after restriction digestion based on size via terminal restriction fragment length polymorphism analysis (10). The number of unique sequences or bands detected by these methods is often considered a proxy for organismal diversity. Rather, due to intra-genomic 16S rRNA heterogeneity, these methods are more accurately a measure of 16S rRNA sequence diversity. Similarity, intra-genomic sequence heterogeneity limits the phylogenetic resolution of the 16S rRNA gene (2, 5). The majority of 16S rRNA

**TABLE 2.1.** Intragenomic 16S rRNA variability for Bacteria and Archaea with full-genome sequence availability.

| Organism | No. rRNA Operons [a] | diff (nt) [b] | % diff [c] |
|---|---|---|---|
| *Aquifex aeolicus* VF5 | 2 | - | - |
| *Bacillus subtilis* ATCC 23857 | 10 | 1 - 15 | 0.97 % |
| *Campylobacter jejuni* ATCC 700819 | 3 | - | - |
| *Deinococcus radiodurans* ATCC 13939 | 3 | 0 - 2 | 0.13 % |
| *Escherichia coli* ATCC 10798 | 7 | 0 - 19 | 1.23 % |
| *Haemophilus influenzae* ATCC 51907 | 6 | - | - |
| *Helicobacter pylori* 26695 | 2 | - | - |
| *Methanococcus jannaschii* DSMZ 2661 | 2 | 3 | 0.20 % |
| *M. themoautotrophicum* ATCC 29096 | 2 | 2 | 0.14 % |
| *Neisseria menigitidis* MC 58 | 4 | - | - |
| *Treponema pallidum* ATCC 25870 | 2 | - | - |
| *Ureaplasma urealyticum* serovar 3 | 2 | 1 | 0.07 % |
| *Vibrio cholerae* ATCC 39315 | 8 | 0 - 14 | 0.91 % |
| *Xyella fastidosa* 9a5c | 2 | - | - |

[a] Number of rRNA operons per genome.

[b] pairwise difference range between 16S rRNA genes per genome.

[c] pairwise difference range between 16S rRNA genes per genome calculated as a percentage.

-, no nucleotide differences.

entries in public databases, such as GenBank and the Ribosomal Database Project, are "composite" sequences obtained from sequencing PCR amplicons generated through simultaneous amplification of all 16S rRNA gene copies on a genome (3).

In an attempt to understand the evolutionary implications of rRNA operon gene redundancy, our laboratory has maintained an internal database of rRNA operon copy number values for both Bacteria and Archaea. Mapping of this information onto a phylogenetic tree indicates that phylogenetic relatedness is not the sole determinant of rRNA operon copy number (9). Rather, bacteria with the same number of rRNA operons appear to have arisen convergently in several phylogenetic lineages. While our primary interest resides in elucidating the underlying physiological and evolutionary consequences of rRNA operon multiplicity, rRNA operon copy number information has become increasingly valuable to researchers performing emerging technologies such as quantitative real-time PCR (11). Working closely with the Ribosomal Database Project at Michigan State University, we have created an Internet-based interactive database of rRNA operon copy number values for a diverse collection prokaryotic microorganisms: The Ribosomal RNA Operon Copy Number Database (*rrndb*).

## DATABASE DESCRIPTION

The *rrndb* provides information pertaining to the number of rRNA operons contained on the genomes of prokaryotic microorganisms in a phylogenetic context. The *rrndb* is co-located with the RDP server at the Center for Microbial Ecology at Michigan State University and is accessible via the WWW at http://rrndb.cme.msu.edu. The initial release of our database (December 2000) contains over 250 annotated entries, including information from all full-genome sequencing projects completed at the time of release. An internal database management system (described below) permits entry of data from any WWW browser, facilitating public release of information shortly after entry and verification. The *rrndb* WWW site also contains answers to frequently asked questions, an opportunity to provide feedback, and a form for direct submission of new data.

## WEB INTERFACE

Information contained within the rrndb is accessible via three main interfaces 1) 'Operon Sort' – a complete list of organisms in the database presented in alphabetical order, 2) 'Phylo Sort' – rRNA operon copy number mapped onto the RDP organismal hierarchy, and 3) a 'Search' page. The 'Operon Sort' list can be sorted in ascending and descending order by organism name, rRNA operon copy number, or genome size (Figure 2.1). rRNA operon copy number is mapped onto the RDP organismal hierarchy presented on the 'Phylo Sort' page. The hierarchy is expandable and collapsible, and mean rRNA operon copy number is displayed for each phylogenetic group (Figure 2.2). User queries can be entered on the 'Search' page, which also offers advanced searches limited to rRNA operon copy number and genome size. Each entry in the rrndb is linked to an individual page containing detailed information about the selected organism, including: genus, species, sub-species, strain, culture deposit, 16S, 23S, and 5S rRNA gene copy number, phylogenetic position, genome size, genome sequence availability, 16S rRNA sequence records, and literature reference(s). Sequence deposits are linked directly to GenBank and the RDP, culture deposits to the American Type Culture Collection (ATCC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), and references to the National Library of Medicine's PubMed database. 16S rRNA genes from individual rRNA operons are denoted when available and include gene start and stop locations within GenBank entries from full-genome sequence deposits.

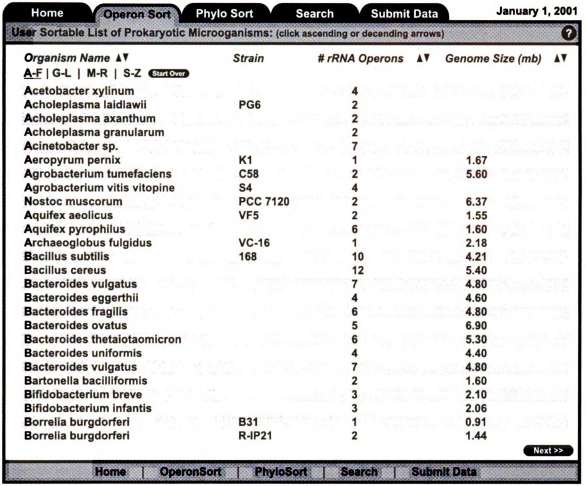


**FIGURE 2.1.** HTML interface of the rrndb. 'Operon Sort' page offering a complete list of database entries; sortable by organism name, rRNA operon copy number, and genome size fields.

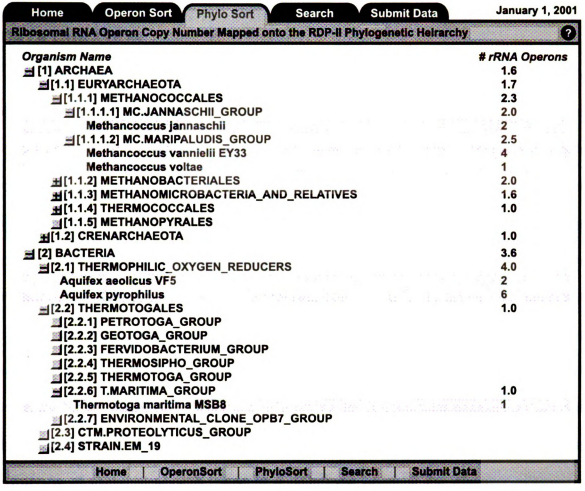


**FIGURE 2.2.** HTML interface of the rrndb. 'Phylo Sort' page with rRNA operon copy number mapped onto the RDP phylogenetic hierarchy. Mean rRNA operon copy number displayed for each phylogenetic group.

## DATA CURATION

All entries in the *rrndb* possess (at minimum) a genus name, strain designation or culture deposit number, and a literature or electronic reference describing rRNA operon copy number determination. The entries for each organism are obtained from computerized searches of reference databases (PubMed, ISI Current Contents, etc.), literature articles, full genome sequencing projects listed at The Institute for Genomic Research (http://www.tigr.org) and the National Center for Biotechnology Information's websites (http://www.ncbi.nlm.nih.gov), and from direct website submission. Effort is made to include all pertinent references to the determination of rRNA operon copy number for an organism. Literature references for a particular organism may not be reported due to an article predating electronic database records, or to the absence of relevant search terms in a database entry. If the complete genome sequence of an organism is available, those data are considered to be the most accurate determination of rRNA operon copy number and is the only reported reference. In certain instances the 16S, 23S, and 5S rRNA genes are not present in equal numbers per genome (8). Laboratory methods to determine rRNA operon copy number typically rely upon Southern hybridization of a 16S rRNA-based probe to restriction-digested genomic DNA; in these instances, the number of 16S rRNA genes serves as an estimate for rRNA operon copy number.

## DATABASE MANAGEMENT SYSTEM

The *rrndb* data are stored using the MySQL relational database management system (RDBMS), which supports the structured query language (SQL) standard (http://www.mysql.com) (Figure 2.3). The WWW interface to the *rrndb* is generated by Java Server Pages and Java Servlets that retrieve information to be displayed by employing custom designed JavaBean objects (http://java.sun.com). These objects access the database using MM MySQL JDBC drivers (http://www.worldserver.com/mm.mysql). The *rrndb* website is hosted on a Sun Ultra 60 server running the Solaris 2.6 operating system and Apache Software Foundation's Apache HTTP and Tomcat servers (http://jakarta.apache.org).

## FUTURE CHANGES & ADDITIONS

Planned additions to the *rrndb* include interface tools to select and download individual organism entries from both the 'Operon Sort' list and the 'Phylo Sort' pages. Information on intra-genomic rRNA sequence variability, such as presented in Table 2.1, will be added for organisms with full genome sequences. Further changes will be dictated by feedback obtained from users of the *rrndb* website. It is anticipated that the *rrndb* will be updated on a quarterly basis as new information becomes available through electronic databases and full-genome sequencing projects.

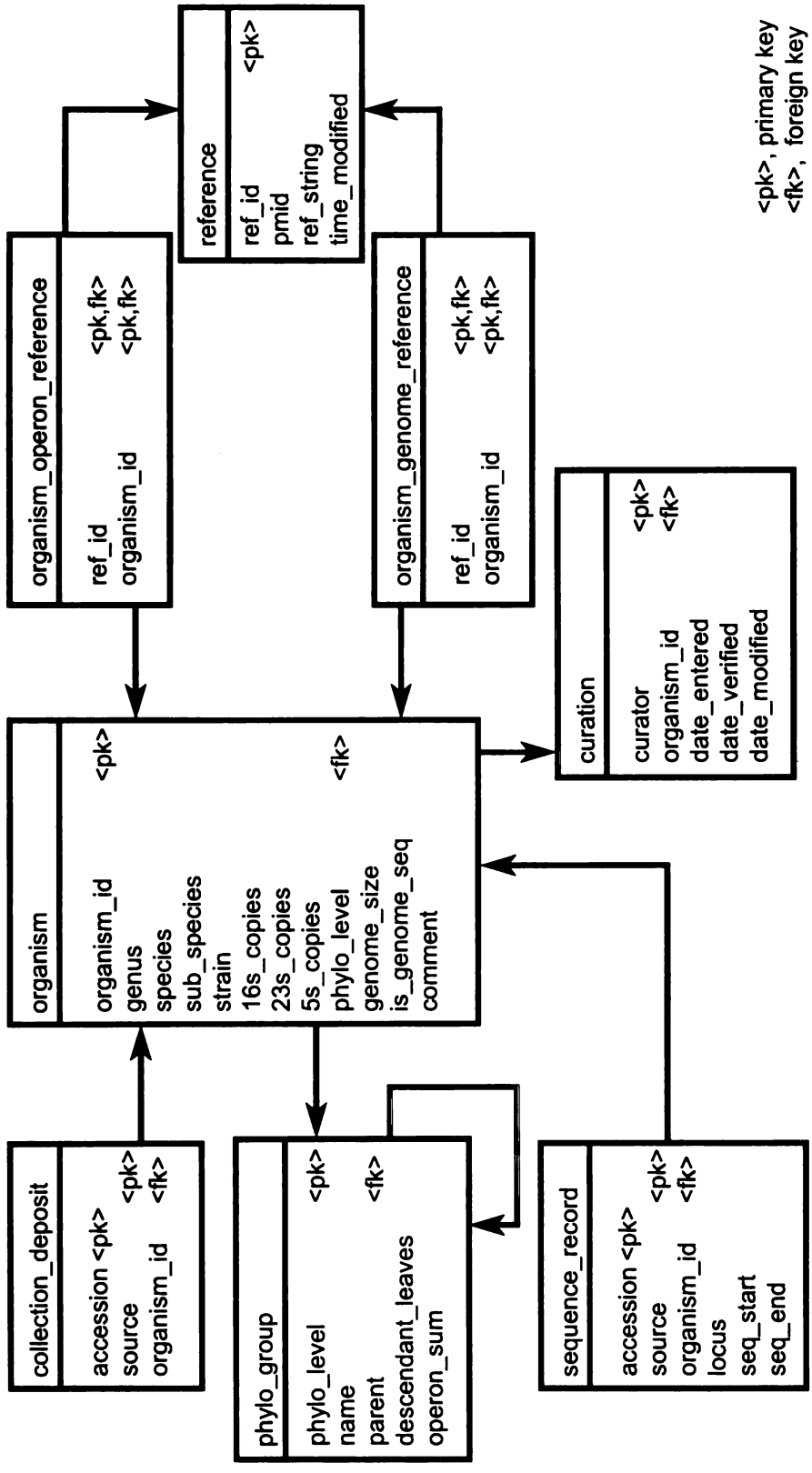


**FIGURE 2.3.** Relational Database Schema.

## ACKNOWLEDGEMENTS

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# CHAPTER 3

## RIBOSOMAL RNA OPERON COPY NUMBER REFLECTS ECOLOGICAL STRATEGIES OF BACTERIA

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## INTRODUCTION

Genes encoding the 5S, 16S, and 23S rRNAs are typically organized into an operon in the *Bacteria*. The number of rRNA operons per bacterial genome varies from 1 to as many as 15 copies (28). For example, the pathogenic bacteria *Rickettsia prowazkeii* (2) and *Mycoplasma pneumonia* (4) have one rRNA operon, while the enteric bacteria *E. coli* (12) and *Salmonella typhimurium* (1) each possess seven copies per genome. The highest number of rRNA operons per genome known can be found among spore-forming bacteria isolated from soil; *Bacillus subtilis* (23) and *Clostridium paradoxum* (28) possess 10 and 15 copies, respectively. Several hypotheses have been proposed to explain the wide variation observed in rRNA operon copy number.

It is generally assumed that multiple copies of rRNA operons in prokaryotic organisms are required to achieve high growth rates. However, the short doubling time observed for certain bacteria with a single rRNA operon (36), and the marginal impact of rRNA operon inactivation on maximal growth rate (8, 27) suggests that the capacity for rapid growth is not the sole determinant of rRNA operon copy number. The number of transcripts that can be initiated at a rRNA operon promoter and the transcriptional rate of RNA polymerase set a maximum rate on the number of ribosomes that can be produced from a single rRNA operon. Calculations including promoter initiation efficiency and transcription rates indicate that one copy of the rRNA operon is insufficient to supply the number of ribosomes required to achieve maximal growth rates observed in *E. coli* (5).

Given the high demand for rRNA transcription and the central role of rRNAs in the regulation of ribosome synthesis, it is conceivable that the number of rRNA operons may dictate the rapidity with which microbes can synthesize ribosomes and respond to favorable changes in growth conditions (8, 29). Transcription of the rRNA operon is regulated to correspond with resource availability and can represent as much as 70% of total cellular transcription during rapid periods of growth (6). A proposed homeostatic model of ribosome biosynthesis provides a direct link between resource availability and the protein synthetic capacity of a bacterial cell (15). The concentrations of resources available for growth determine intracellular concentration of ATP and GTP (adenosine- & guanosine triphosphate), which in turn regulate the efficiency of transcription initiation at rRNA operons. The rRNA operon transcript is processed enzymatically to yield mature rRNAs that not only bind ribosomal proteins during assembly of the ribosome, but also regulate translation of the ribosomal protein mRNAs (35). In *E. coli*, a positive relationship exists between the number of rRNA operons inactivated and the time required to increase growth in response to added resources (8). Condon et al. (8) suggested that *E. coli* maintains seven rRNA operons due to selective pressure on the ability to adapt quickly to environmental conditions. Therefore, the capacity to respond rapidly to fluctuating growth conditions may be more relevant than maximal growth rate when explaining the variation in rRNA operon multiplicity in different species of bacteria.

While multiple rRNA operons may provide an advantage under fluctuating conditions, constitutive expression from multiple rRNA operons would confer a metabolic expense on slower growing cells due to the overproduction of ribosomes. Extra copies of plasmid-borne rRNA operons increase stable RNA concentrations while concomitantly decreasing growth rates in *E. coli*, indicating a potential cost associated with constitutive expression from multiple rRNA operons at slow growth rates (29). The immediate degradation of ribosomes in starved cells of *E. coli* and *Salmonella* spp. also suggests that excess translation capacity is metabolically unfavorable in conditions of low-nutrient availability (9, 20). For these reasons we postulate that fewer rRNA operons represent a competitive advantage at slow growth rates.

The observations above led us to question whether the number of rRNA operons in phylogenetically diverse bacteria reflected ecological strategies characterized by either rapid response to resource input (high copy number) or efficient allocation of resources under constant, slow growth environments (low copy number). To determine whether the number of rRNA operons is of adaptive significance to bacteria rather than the result of genetic drift or coincidence, we explored the relationship between rRNA operon copy number, organismal phylogeny, and the capacity of bacteria to respond to added resources. Microbes isolated from soil were selected for this study because soils are inhabited by a rich diversity of microbes (32) and the soil matrix provides an array of micro-environments that can vary considerably with regard to resource availability. If rRNA operon copy number reflects the ecological strategy of

bacteria in response to resource availability, bacterial populations with different numbers of rRNA operons are likely to coexist in soils and respond differently to perturbations.

## MATERIALS & METHODS

### Colony Response Curves of Soil Bacteria

Soil samples used for experimentation were obtained from the Long Term Ecological Research (LTER) site at Kellogg Biological Station, Hickory Corners,, Michigan in May 1997. Soil cores (10 cm depth x 2.5 cm diameter) were removed from five locations within a conventional-till agricultural plot (plot T1, descriptions of plots may be accessed at http://lter.kbs.msu.edu). Sample cores were sieved (2 mm mesh), homogenized, and stored on wet ice for no more than six hours prior to utilization. A 100 g portion of homogenized soil was suspended in 1.0 L of 5 mM $K_2HPO_4$ buffer (pH 7.0), shaken (22° C, 150 rpm, 15 min), and a 1 mL portion of the suspension was serially diluted. Aliquots from the dilution series were plated on 1.5% agar medium containing a 100–fold dilution of Nutrient Broth (Difco Inc., Detroit) and bacterial colonies were enumerated at periodic time intervals and marked for subsequent isolation upon completion of the colony response curve. The laboratory of T. Hattori has demonstrated that the pattern of colony formation by soil bacteria is reproducible and can be modeled by the super-imposition of several (typically four) first-order reaction curves (16, 19). Bacteria from the time intervals designated 'I' and 'IV' (see Figure 3.1; groups described in ref (16) were isolated and characterized for rRNA

operon copy number. Single colonies were picked and streaked for isolation a minimum of six times on dilute Nutrient Agar plates (Difco, Inc.); culture purity was also confirmed via light microscopy and PCR amplification of 16S rDNA (see below). Isolates from rice paddy soils were obtained from Dr. Tsutomu Hattori (Institute of Genetic Ecology, Tohoku University, Sendai, Japan)(16, 26).

**rRNA Operon Copy Number Determination for Soil Isolates**

Genomic DNA was obtained from each soil isolate, independently digested with at least three different restriction enzymes (*Acc*I, *Bst*EII, *Pin*A1, *Pvu*II, *Pst*I, or *Sac*I; Gibco/BRL Co.), and separated on a 1.0% agarose gel using standard methods (3, 25). Ribosomal RNA operon copy numbers were determined by Southern hybridization analysis of gel-separated restriction digests using a digoxigenin-dUTP-labeled DNA probe complementary to a conserved region (positions 8 - 536) of the *E. coli* 16S rDNA gene. Alternative arrangements of rRNA genes into operons are known, but individual rRNA genes are usually present in stoichiometric quantities. Therefore, the number of 16S rRNA gene copies (number of bands with equal intensity hybridizing to the 16S rDNA probe) was considered a reasonable estimator of the number of rRNA operon equivalents per genome. In cases where enzymatic digestion failed to resolve hybridized fragments or bands of equal intensity could not be discriminated, results were discarded and additional analyses were performed with different restriction endonucleases. Genomic DNA isolated from *E. coli* and digested with *Pvu*II was included on Southern hybridization gel as a positive control.

**Phylogenetic Analyses**

The 16S rDNA gene was amplified from early and late appearing soil isolates using primers 8f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') with reaction conditions described previously (22, 34). Partial sequences were obtained from soil isolates with an automated DNA sequencer (ABI 373A) using fluorescent dideoxy dye terminator chemistry and overlapping sequencing primers 8f and 519r (5'-GTATTACCGCGGCTGCTGG-3'). Sequences were initially aligned using the ARB software package (http://www.biol.chemie.tu-muenchen.de) automated aligner and then verified manually against known secondary structures (30). Soil isolate partial sequences (between positions 28 – 519 of the *E. coli* 16S rDNA consensus) were added to a Ribosomal Database Project (24) sub-tree using parsimony with the ARB software package (30). Branch lengths were right aligned for presentation purposes in ARB and therefore do not necessarily represent the actual evolutionary distance, but the branching topology is preserved.

**Soil Microcosm Amendment Experimentation**

Soil microcosms were established from homogenized soil collected from the top 10 cm of a fallow agricultural plot (LTER, Kellogg Biological Station) which had no previous documented exposure to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). Ten to fifteen soil samples from a 6 $m^2$ area were pooled and sieved (2 mm mesh). For each microcosm, 243 g of soil (8% moisture content) were transferred to a polyethylene bag while 27 g of soil were dried (100°C, overnight) to serve as a carrier for liquid amendments. Each 27 g

portion of carrier soil was mixed with 2,4-D dissolved in 0.1M $Na_2HPO_4$ buffer (pH 7.0) or buffer alone such that each microcosms received identical concentrations of sodium phosphate but either 0, 10, or 100 µg 2,4-D per g soil (final concentration). Final moisture content of soil in each microcosm was adjusted to 25% (w/w) with sterile, distilled water. Microcosms were incubated for one week during which samples were periodically removed for isolation of 2,4-D-degrading bacteria. Colonies able to degrade 2,4-D were identified by autoradiography based on their ability to incorporate $^{14}C$ from $^{14}C$–ring labeled 2,4-D into biomass (10). Ribosomal RNA operon copy numbers were determined as described above with Southern hybridization analysis of genomic DNA digested with *Eco*R1 or *Pvu*II.

**Amplified rDNA Restriction Analysis of 2,4-D-degrading Isolates**

Bacterial species able to degrade 2,4-D were identified based on restriction fragment length polymorphism (RFLP) patterns resulting from gel electrophoresis of enzymatically digested, PCR amplified, 16S rDNA. 16S rDNA was amplified using primers 8f and 1492r and reaction conditions described previously (22, 34). Amplified DNA from isolates was independently digested with *Msp*I, *Cfo*I, *Alu*I, or *Hae*III. Digested DNA was electrophoresed through 2.75% Metaphor agarose gels (FMC Bioproducts, Inc.) and the RFLP patterns of all isolates were compared. Isolates whose 16S rDNA restriction pattern differed with at least one enzyme were defined as different species.

**Nucleotide Sequence Accession Numbers**

The nucleotide sequences for rice paddy isolates have been previously deposited in the GenBank database under accession numbers D84561, D84564, D84568, D84570, D84577, D84597, D84604, D84635, D84639, D84640, D84641, D84644, D84645. Nucleotide sequences for isolates obtained from Kellogg Biological Station Long Term Ecological Research site are deposited in GenBank under sequence accession numbers AF183149 – AF183159.

## RESULTS AND DISCUSSION

In environments with periodic resource fluctuations, lag time (*L*, the time before initiation of cell division) and maximal growth rate ($\mu_{max}$) are important components of fitness (21, 33). Populations that can rapidly achieve high maximal growth rates (short *L*, high $\mu_{max}$) are able to utilize available resources before competing populations. In contrast, lag time does not impose a fitness advantage in environments with a constant supply of resources (18, 31). Multiple rRNA operons allow transcriptional initiation from multiple loci permitting a rapid increase in the intracellular concentration of rRNA, thereby effectively decreasing lag time. A potential tradeoff for a rapid up-shift capacity is the metabolic expense of rRNA overproduction at slow growth rates, apparently due to inadequate regulation of rRNA operons (29). In agreement with these observations, bacteria isolated from low-nutrient aquatic environments share the characteristics of slow growth rate and few (typically 1 – 2) rRNA operons (7, 13).

**Response Time of Soil Isolates and rRNA Operon Copy Number**

To test whether rRNA operon copy number is correlated with the response time (a function of $\mu_{max}$ and *L*) of bacterial populations in soil to resource availability, heterotrophic bacteria appearing early and late on agar media were isolated from soils from an agricultural research site in Michigan and a rice paddy near Sendai, Japan (16) (Figure 3.1, A). Early appearing isolates possessed, on average, a significantly greater number of rRNA operons ($\bar{x}$ = 5.5 copies) than late appearing isolates ($\bar{x}$ = 1.4 copies)(Figure 3.1, B). Of the early-appearing isolates, 6 of 11 contained 5 or more copies of the rRNA operon per genome, while 12 of 13 late appearing species contained 2 or fewer copies. The time required for colony formation was a phenotype retained by isolates upon subsequent transfer on solid media (personal observation and ref (26)). The biased distribution of diverse soil bacteria with high rRNA operon copy numbers appearing early on two different complex culture media suggests that the response of bacteria to favorable growth conditions reflects ecological strategies and not solely the ability to utilize a particular limiting resource.

The ability of bacteria with high rRNA operon copy number to rapidly respond to nutrient enrichment likely influences their population dynamics in soil (discussed below) and their recovery from soil using common enrichment techniques. Our capacity to culture only a small proportion of the diversity in soil (estimated between 0.1 – 0.5%;(32) may result from the inability of bacteria, with low rRNA operon copy number, to form visible colonies in a short period of time.

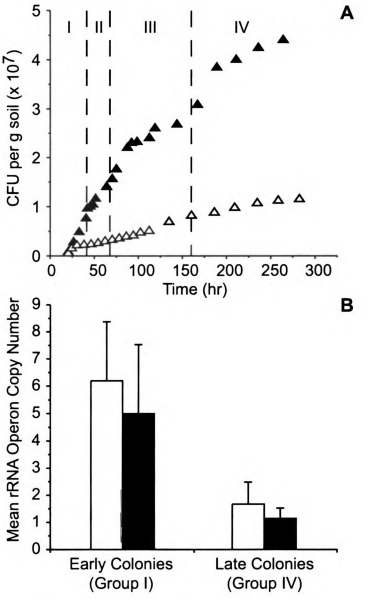


**FIGURE 3.1.** Correlation between time of colony appearance and rRNA operon copy number. **(A)**, Colony appearance curve for soil isolates. Open triangles (Δ) correspond to colonies from conventional-tilled agricultural soil in Michigan; filled triangles (▲) from rice paddy soils in Japan (adapted from ref. 16). Each point represents the arithmetic average of colonies observed on a minimum of three agar plates at that time interval. Bacteria from the time intervals designated 'I' and 'IV' (groups described in ref. 16) were isolated and characterized for rRNA operon copy number. **(B)**, Mean number of rRNA operons for bacterial isolates from group I (early colony formers) and group IV (late colony formers) are presented as: rice paddy isolates (filled boxes: $n$ = 6, early; $n$ = 7, late), conventional-tilled soil isolates (open boxes: $n$ = 5, early; $n$ = 6, late). Error bars are one standard deviation above the sample mean.

**Relationship of rRNA Operon Copy Number to Phylogeny and Genome Size**

The phylogeny of isolates was reconstructed to preclude the possibility that the number of rRNA operons per isolate reflected evolutionary history alone. A statistical correlation between rRNA operon copy number and response time assumes that species were removed independently from the same distribution (14). Since bacteria were isolated based on the phenotypic parameter of response time, it was possible that the correlation with rRNA operon copy number resulted from two monophyletic groups of bacteria with either high or low numbers of rRNA operons per genome. The distribution of rRNA operon copy number within a collection of phylogenetically diverse bacteria indicated no obvious evolutionary constraint on the number of rRNA operons per genome (Figure 3.2). The occurrence of bacteria with the same number of rRNA operons in disparate phylogenetic lineages appears to have arisen from convergent evolution, driven by adaptation to similar selective pressures influencing the fitness of bacteria in different environments.

**FIGURE 3.2.** Phylogenetic distribution of bacteria characterized for rRNA operon copy number. Filled boxes indicate soil isolates that appeared early, while open boxes indicate isolates that appeared late. Isolates from conventional-tilled soils in Michigan (designated by "KBS-") and rice paddy soils in Japan (designated by "HF-" or "HS-") are included. Values to the right of species' names indicate the number of rRNA operon equivalents per chromosome. Major phylogenetic divisions are indicated on the far right with abbreviations as follows: C/F/B = Cytophaga/Flexibacter/Bacteroides, CYN = cyanobacteria, SPR = spirochetes, TRM = thermophiles. Strain designations and literature references for 16S rRNA sequences and rRNA operon copy numbers used in this figure are available at the Ribosomal RNA Operon Copy Number Database (http://rrndb.cme.msu.edu).

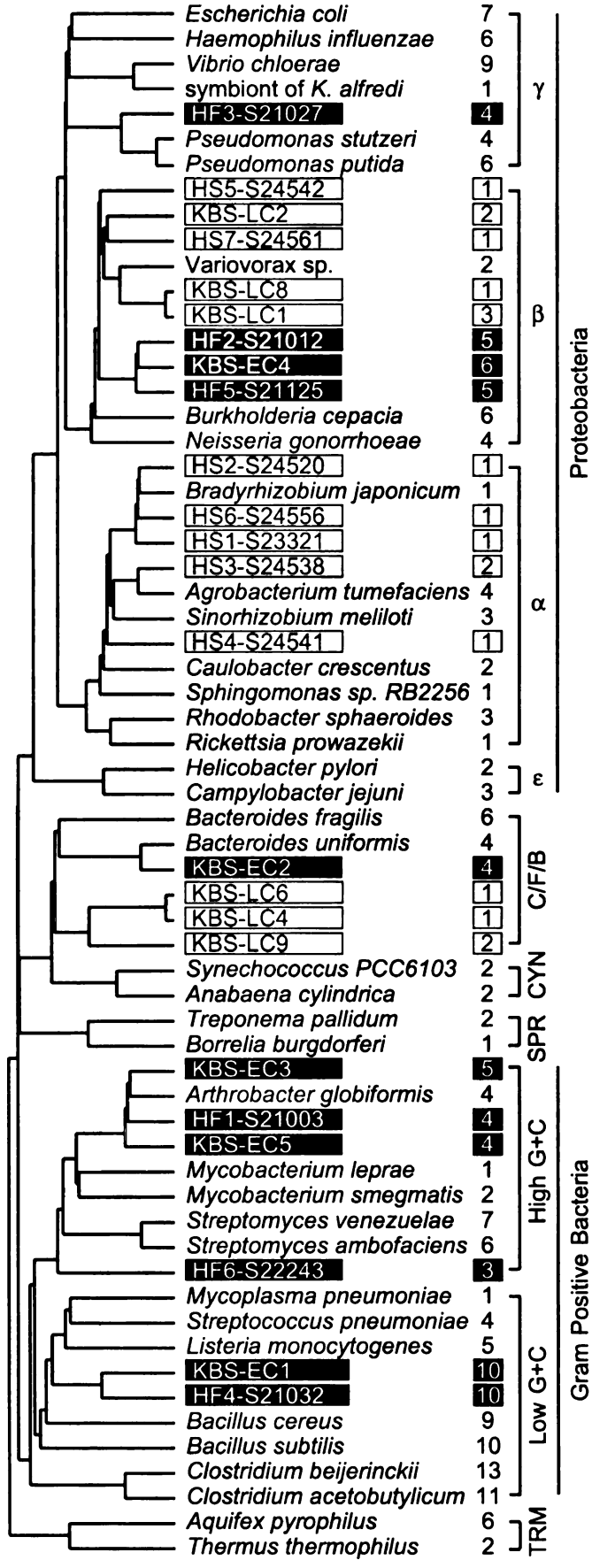
Escherichia coli 7
Haemophilus influenzae 6
Vibrio chloerae 9
symbiont of K. alfredi 1
HF3-S21027 4
Pseudomonas stutzeri 4
Pseudomonas putida 6
HS5-S24542 1
KBS-LC2 2
HS7-S24561 1
Variovorax sp. 2
KBS-LC8 1
KBS-LC1 3
HF2-S21012 5
KBS-EC4 6
HF5-S21125 5
Burkholderia cepacia 6
Neisseria gonorrhoeae 4
HS2-S24520 1
Bradyrhizobium japonicum 1
HS6-S24556 1
HS1-S23321 1
HS3-S24538 2
Agrobacterium tumefaciens 4
Sinorhizobium meliloti 3
HS4-S24541 1
Caulobacter crescentus 2
Sphingomonas sp. RB2256 1
Rhodobacter sphaeroides 3
Rickettsia prowazekii 1
Helicobacter pylori 2
Campylobacter jejuni 3
Bacteroides fragilis 6
Bacteroides uniformis 4
KBS-EC2 4
KBS-LC6 1
KBS-LC4 1
KBS-LC9 2
Synechococcus PCC6103 2
Anabaena cylindrica 2
Treponema pallidum 2
Borrelia burgdorferi 1
KBS-EC3 5
Arthrobacter globiformis 4
HF1-S21003 4
KBS-EC5 4
Mycobacterium leprae 1
Mycobacterium smegmatis 2
Streptomyces venezuelae 7
Streptomyces ambofaciens 6
HF6-S22243 3
Mycoplasma pneumoniae 1
Streptococcus pneumoniae 4
Listeria monocytogenes 5
KBS-EC1 10
HF4-S21032 10
Bacillus cereus 9
Bacillus subtilis 10
Clostridium beijerinckii 13
Clostridium acetobutylicum 11
Aquifex pyrophilus 6
Thermus thermophilus 2
γ
β
α
ε
Proteobacteria
C/F/B
CYN
SPR
High G+C
Low G+C
Gram Positive Bacteria
TRM

**FIGURE 3.2**

A simple relationship is also not evident between the number of rRNA operons and genome size as might be expected if recombination between rRNA operons leads to increases in genome size (1, 17), or if multiple rRNA operons are required to provide translation capacity for an increased number of protein-encoding genes in larger genomes. A linear regression provides only weak evidence for a positive correlation between genome size and rRNA operon copy number (Figure 3.3). While the ability to explain changes in genome size based on increased rRNA operon copy number is low ($R^2 = 0.12$), the hypothesis that no relationship exists between these variables cannot be rejected ($P < 0.01$). It is possible that the strength of this relationship is biased towards easily cultivable bacteria or those that are easy to manipulate genetically. One group in particular that may weaken the relationship between genome size and rRNA operon copy number are the limited number of bacteria ($n = 4$) with greater than 10 rRNA operons (Figure 3.3).

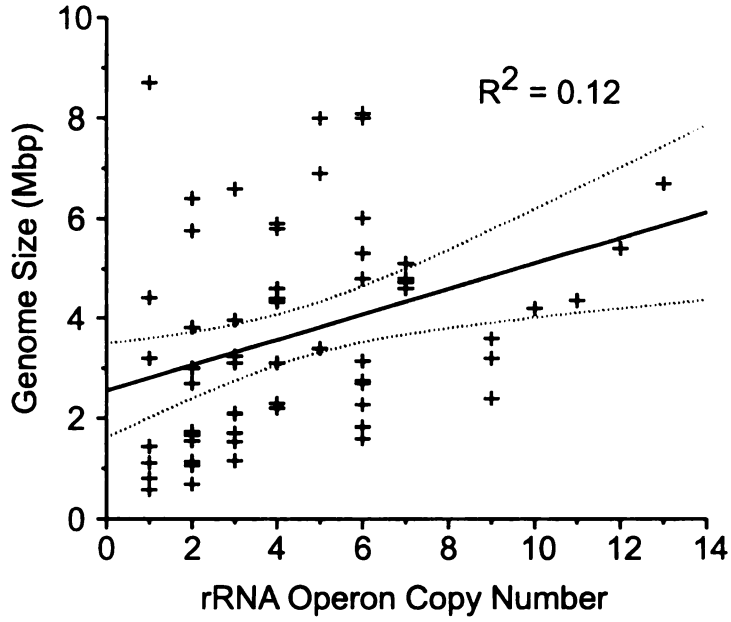


**FIGURE 3.3.** The relationship between genome size and rRNA operon copy number. Phylogenetic taxa represented: *Proteobacteria* classes alpha (n = 8), beta (n = 3), gamma (n = 9), epsilon (n = 4), Cytophaga/Flexibacter/Bacteriodes (n = 7), *Cyanobacteria* (n = 4), spirochetes (n = 4), high G+C Gram positive bacteria (n = 7), low G+C Gram positive bacteria (n = 16), thermophiles (n = 3). A linear regression for all data points was calculated using the least-squares method ($P < 0.01$ that no relationship exists); upper and lower 95% confidence bounds are indicated by dotted lines. Data used in this figure is available at the Ribosomal RNA Operon Copy Number Database (http://rrndb.cme.msu.edu).

**Effects of Selection in Soil Microcosms**

The potential adaptive significance of rRNA gene copy number was tested directly in soil microcosms by examining the dynamics of indigenous bacterial populations competing for the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). The relative abundance of bacterial populations able to use 2,4-D as a sole carbon and energy source was measured before and after selection in nine soil microcosms (11). In unamended microcosms, there were 1 $x10^3$ colony forming units (cfu) of 2,4-D-degrading bacteria per g soil. Not surprisingly, the total number of 2,4-D-degrading bacteria increased to 1 x $10^5$ and 1 x $10^6$ over seven days following a one-time pulse with either 10 or 100 ppm 2,4-D (final concentration), respectively (11). Although there was a dramatic change in the number of 2,4-D-degrading bacteria, the total number of readily cultured bacteria remained at approximately 1 x $10^7$ cfu/g soil in each of the 9 microcosms. A total of 837 isolates representing 38 2,4-D-degrading species were isolated from the microcosms. The effect of selection for rRNA operon copy number among 2,4-D-degrading populations is clearly apparent between unamended and amended microcosms (Figure 3.4). The most abundant species in unamended microcosms were minor components of microcosms amended with 10 or 100 ppm 2,4-D, while several species at low abundance in the controls developed into numerically dominant populations in the pulsed microcosms.

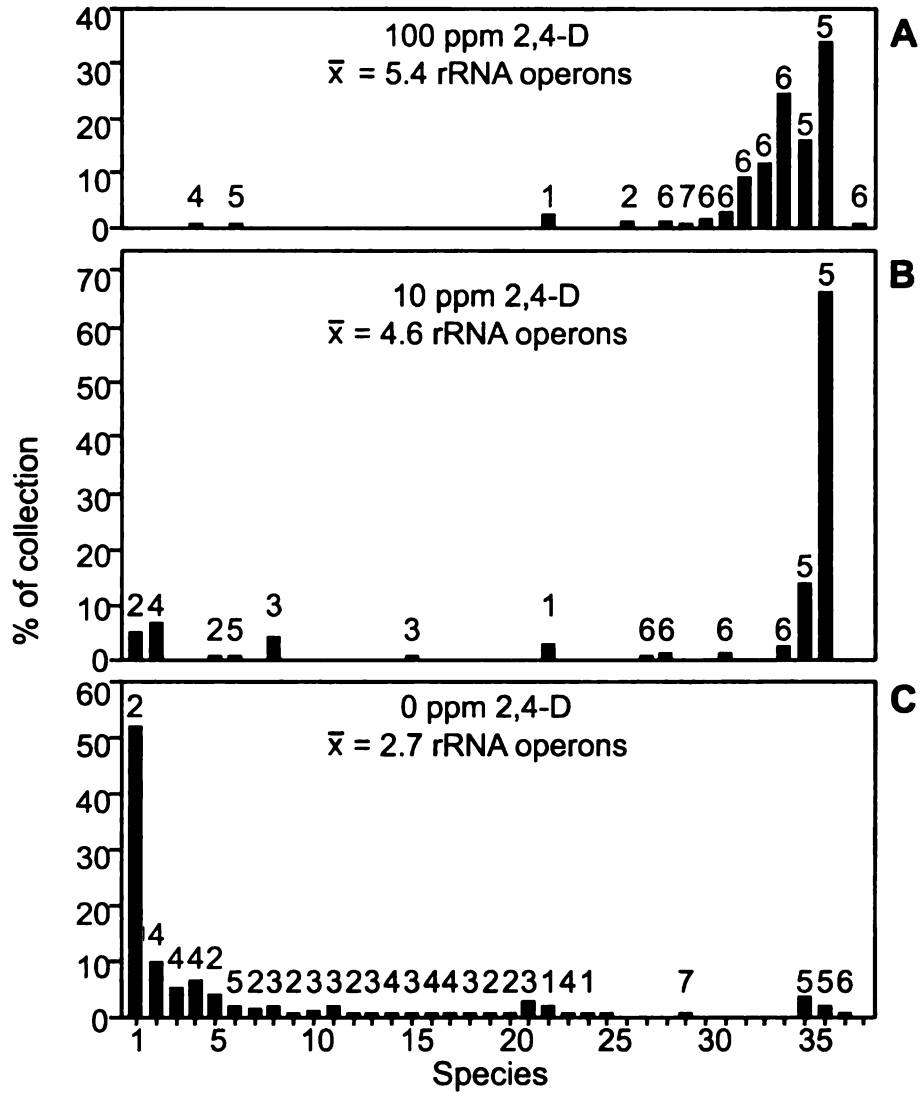


**FIGURE 3.4.** The distribution of ribosomal RNA operon copy number among 2,4-D-degrading bacteria isolated from amended and unamended soil microcosms. Species are identified based on similarity of 16S rRNA restriction patterns. The height of each bar reflects the abundance of that species relative to all isolates from that microcosm (**A**) 247 isolates. (**B**), 263 isolates, (**C**) 327 isolates. Numbers above bars indicate the rRNA operon copy number for each species; the mean number of rRNA operons per isolate is indicated for each treatment. Different isolates of the same species exhibited similar rRNA operon copy numbers (data not shown). Each treatment represents data from three replicate microcosms

.

The majority of 2,4-D-degrading bacteria contained between 1 and 4 rRNA operons in unamended microcosms (Figure 3.4, C). In contrast, among the species detected in microcosms amended with 10 or 100 ppm 2,4-D, 7 of 13 and 11 of 14 species, respectively, possessed between 5 and 7 rRNA operon copies (Figure 3.4, A & B). Despite a reduction in species diversity, the effect of selection for 2,4-D-degrading species with higher rRNA operon copy number in amended microcosms is significant with ($P < 0.001$) or without ($P < 0.01$) consideration of species abundance. (A one-tailed Student's t-test assuming unequal sample variance was used to test the hypothesis that selection for higher rRNA operon copy number was greater in the microcosms receiving 10 ppm and 100 ppm 2,4-D amendment than in the unamended (0 ppm) microcosms. A non-parametric (Wilcoxon ranked-sum) test yielded similar results ($P < 0.02$) when population abundance was excluded from the analysis to reduce the contribution of highly abundant species.)

The positive correlation between rRNA operon copy number and 2,4-D concentration supports an association between rRNA operon copy number and competitive fitness. In fact, the ability of 2,4-D-degrading populations with high rRNA gene copy number to respond rapidly to new resource conditions was a general characteristic of these populations as demonstrated by comparison of the growth rates in liquid media of a number of 2,4-D-degrading populations on a variety of substrates (succinate or acetate as the limiting carbon and energy source, and in complex medium) other than 2,4-D (data not shown).

## CONCLUSIONS

Experiments described above elucidate the potential role of rRNA operon multiplicity by providing a direct correlation between rRNA operon copy number and the time required for soil bacteria to form colonies (a function of $\mu_{max}$ and $L$) in response to resource availability. The potential adaptive significance of rRNA operon multiplicity was demonstrated in soil microcosms by the reproductive success of diverse 2,4-D-degrading bacteria containing a significantly greater number of rRNA operons per genome during competition for a pulse of 2,4-D. We propose that the number of rRNA operons in a bacterial genome represent one trait among a group of interdependent traits that comprise a strategy for responding to the availability of resources.

As genomic information rapidly accumulates for the *Bacteria* from whole genome sequencing projects, our models of bacterial competitiveness becomes increasingly complex as individual genes are considered in the context of the entire genome and, ultimately, the organism. Certainly no single gene product can determine bacterial competitiveness in all environments. However, gene products involved in the regulation of central metabolism and cellular growth may establish a basic foundation for the competitive success of a bacterial species. Genes directly involved in the response of bacteria to specific selective pressures from the environment will undoubtedly further shape the competitive fitness, or life history strategy of a species. The correlation between the copy number of rRNA genes and the response rate of diverse bacteria to a variety of growth substrates indicates an evolutionary linkage between the number of rRNA genes

and the basic competitive ability of bacterial species. While other genes may enhance this basic ability, the multiplicity of rRNA genes in the Bacteria provides a genetic indicator of the general ecological strategy of a bacterial species for exploitation of nutrients.

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# CHAPTER 4

## SHORT-TERM RESPONSE OF HETEROTROPHIC SOIL BACTERIA TO CARBON AND WATER AMENDMENT AS PREDICTED BY RIBOSOMAL RNA OPERON COPY NUMBER

## INTRODUCTION

Microbial communities in soils and sediments are the most abundant and phylogenetically diverse biological assemblages on Earth. Microbial communities are critical to the cycling of organic matter and catalyze unique biochemical transformations necessary for global cycling of carbon and nitrogen (2). Despite the diversity and abundance of soil bacteria (or perhaps more likely as a consequence), we are only beginning to understand the structure and dynamics of these microbial communities. Quantitative differences in rates of microbially mediated processes in soil indicate spatial and resource heterogeneity are important factors affecting microbial community function (3, 25, 31, 32). The diversity of microorganisms carrying out specific microbial processes can also contribute to the functionality of the soil ecosystem (12, 16). In grassland communities competing for shared limiting resources, ecosystem stability and productivity are positively related to both species composition and total community diversity (41). Whether community diversity and species composition similarly affect the stability and productivity of soil microbial communities is largely unknown (43). Linking microbial community structure and function requires an ecological understanding of individual microbial populations and the phylogenetic scale at which microbial diversity is relevant to ecosystem function.

Changes in soil water content are one of the most frequent disturbances affecting soil microbial communities, yet our understanding of this phenomenon is primarily limited to the process level. Metabolic activity of heterotrophic soil

microorganisms (inferred from biomass $CO_2$ respiration) is a direct function of water content, which facilitates transport and availability of growth-limiting nutrients (37). Rapid changes in soil water content are associated with bursts of microbial respiration that have been attributed to the mobilization of non-living organic material, biomass derived from cellular lysis, and solutes excreted by cells achieving intracellular osmotic balance (22). Since organic matter in soil is largely composed of plant-derived compounds (cellulose, lignin) that are generally recalcitrant to rapid degradation (6), nutrients made available during rewetting are largely attributed to cellular lysis and solute excretion (26, 42). Rapid increases in biomass respiration rates from soils amended with sugars and carboxylic acids provides evidence for both carbon limitations in soil and the enormous metabolic capacity of soil microbial communities (15, 18).

One predictor of microbial response to resource availability is the number of rRNA operons present in a bacterial genome. Soil bacteria with many rRNA operons ($\bar{x}$ = 5.5) can be distinguished by their ability to rapidly form colonies on complex-dilute agar media, whereas bacteria possessing fewer rRNA operons per genome ($\bar{x}$ = 1.4) form colonies more slowly (Chapter 3). rRNA genes are typically organized into operons in the *Bacteria* and can be present from 1 to 15 copies in a single genome (Chapter 2). Bacteria with identical rRNA operon copy numbers are found in distantly related phylogenetic groups on the universal tree, indicating that rRNA operon copy number is not solely limited by evolutionary ancestry (36). For instance, both the α-proteobacteria and *Planctomycetes*

**TABLE 4.1.** Phylogenetic conservation of rRNA operon copy number among microbial groups commonly found in soil.[a]

| RDP Hierarchical Group[b] | Mean | 95% CI[c] |
|---|---|---|
| **[2.15] Cytophaga-Flexibacter-Bacteroides** | | |
| [2.15.1] Bacteroides Group (*n* = 8) | 5.3 | 4 - 6 |
| [2.15.6] Cytophaga Group (*n* = 1) | 1.0 | - |
| **[2.20] Planctomyces & Relatives** (*n* = 7) | 1.3 | 1 - 2 |
| **[2.28] Proteobacteria** | | |
| [2.28.1] Alpha-Subdivision (*n* = 27) | 2.3 | 2 - 3 |
| [2.28.2] Beta-Subdivision (*n* = 7) | 3.9 | 3 - 5 |
| [2.28.3] Gamma-Subdivision (*n* = 64) | 5.2 | 5 - 6 |
| **[2.30] Gram Positive Bacteria** | | |
| [2.30.1] Actinobacteria (high mol%G+C) (*n* = 26) | 4.3 | 4 - 5 |
| [2.30.1.8] Streptomyces Subdivision (*n* = 13) | 5.9 | 6 |
| [2.30.1.13.1] Mycobacteria Group (*n* = 5) | 1.4 | 1 - 2 |
| [2.30.7] Bacillus & Relatives (low mol%G+C) (*n* = 38) | 4.2 | 3 - 5 |
| [2.30.7.9-12] Bacillus-Staphylococcus Groups (*n* = 4) | 9.0 | 7 - 12 |
| [2.30.7.17] Lactobacilli (*n* = 26) | 2.8 | 2 - 3 |
| [2.30.7.21] Streptococci (*n* = 8) | 6.0 | - |
| [2.30.9] Clostridium & Relatives (*n* = 3) | 11.3 | 10 - 13 |

[a] Heirarchical groups contain all organisms categorized in the Ribosomal RNA Operon Copy Number Database, release 2.1

[b] Ribosomal Database Project release 8.0 hierarchical groups (http://rdp.cme.msu.edu)

[c] 95% confidence interval (CI) about the group rRNA operon copy number mean rounded to the nearest integer value.

-, no information available for group or not applicable

taxonomic groups contain species that typically possess less than three rRNA operons per genome (Table 4.1). The correlation between phylogeny and rRNA operon copy number is evident among phylogenetic groups including strains, species, genera, and higher taxonomic levels (Table 4.1, see also Chapter 2). Bacteria phylogenetically related at these phylogenetic levels likely possess similar numbers of rRNA operons per genome.

Synthesis of rRNA in a bacterium is limited both by the rate of rRNA gene transcription and rRNA gene copy number (7). During rapid growth, transcription of the ribosomal RNA operons can account for up to 70% of total cellular transcription and consequently represents the single largest energy expenditure of a bacterial cell (8). A positive relationship between cellular rRNA concentration and growth rate suggests that bacteria with multiple rRNA operons are capable of achieving high maximal growth rates. However, the marginal effect of rRNA operon inactivation on maximal growth rate demonstrates that while rRNA operon copy number sets an upper limit on rates of rRNA synthesis, all rRNA operons are not transcribed at maximal rates (14, 27). rRNA operon inactivation in *E. coli* demonstrates that multiple rRNA operons confer the ability to rapidly shift-up growth rates in response to more favorable growth conditions (14). Therefore, bacteria with multiple rRNA operons may possess an advantage in environments with fluctuating nutrient availability. Conversely, multiple rRNA operons may confer a metabolic cost during slow growth or starvation due to constitutive transcription of the rRNA genes (40).

Experimentation described below explores the relationship between rRNA operon copy number and the response of soil bacterial communities to amendment with succinate and water. It was hypothesized that heterotrophic soil bacteria with multiple rRNA operons possess an increased capacity for rRNA synthesis thereby permitting rapid increases in cellular rRNA content and population size following amendment. Bacterial populations capable of rapidly increasing rRNA synthesis and population size would include phylogenetic groups of bacteria possessing many (≥4) rRNA operons, including the *Actinobacteria*, and γ- & β- classes of the *Proteobacteria* (Table 4.1). Alternatively, little or no increase in rRNA abundance or population size would occur in bacterial populations with few (≤3) rRNA operons, such as the *Planctomycetes* and α-class of the *Proteobacteria* (Table 4.1). Initial experimentation utilized colony appearance time on solid media, containing succinate as a sole source of carbon and energy, to identify dominant soil bacterial populations with many and few rRNA operons. Secondly, soil microcosms were constructed to monitor changes in rRNA abundance and population size in the soil microbial community in response to amendment with succinate. Changes in rRNA abundance were assessed by quantitative rRNA hybridization of total RNA extracted from soil microcosms. Changes in the bacterial population structure were monitored via direct microscopic counts and abundance of early and late appearing colonies on colony appearance curves.

## MATERIALS & METHODS

### Colony Appearance Curve on Succinate Minimal Medium

Soil used for colony appearance curves was obtained from the Long Term Ecological Research (LTER) site at Kellogg Biological Station, Hickory Corners, Michigan on October 9, 1998. The dominant soil type at the KBS-LTER is a fine-to-coarse loamy Hapludalf of moderate fertility and mild-acidity (31). Soil cores (5 cm depth x 1 cm diameter) were obtained from 5 sampling locations within a 1-ha plot receiving conventional agricultural management (a detailed site description can be accessed at http://lter.kbs.msu.edu). Soil cores were pooled, sieved (2 mm mesh), and stored at 4°C for approximately 5.5 hours prior to use. Soil moisture content was determined gravimetrically at the time of sampling. The water holding capacity (WHC) of soil was measured by saturating 10 g of soil with sterile distilled water for 12 hr followed by drying in a microwave oven until a constant weight was recorded after repeated microwave cycles (WHC = (water saturated soil weight – dry soil weight)/dry soil weight) (13).

The appearance of colonies growing on solid media containing succinate as the sole source of carbon and energy was characterized using colony appearance curves (19, 21). Succinate was chosen as a readily utilizable carbon that is known to produce a significant level of microbial biomass respiration when applied to soil (15). A 10 g aliquot of homogenized soil was suspended in 100 mL of sterile soil dilution buffer (5 mM $K_2HPO_4$, 5 mM $MgCl_2$, pH 7.0), mixed for 10 min on a magnetic stirrer, and a 1 mL portion of the suspension was serially

diluted. Dilutions were plated on 1.5% agar medium (Bacto-Agar; Difco, Detroit) containing basal salts (Appendix A) and 5 mM succinate (pH 7.0), and the appearance of bacterial colonies was recorded over 250 hours.

Partial 16S rRNA sequences were used to place selected isolates within bacterial taxonomic groups. Ribosomal RNA operon copy number was determined by gel electrophoretic separation of restriction digested genomic DNA followed by Southern hybridization using a 16S rDNA-based probe (Chapter 3).

**Soil Microcosm Construction**

Soil used for microcosms was obtained from the LTER site at Kellogg Biological Station on March 28, 1999. A 1 kg sample was removed from the top 10 cm of a conventional-till agricultural plot and stored at 4°C for nine days prior to use. After determining initial soil moisture content and water holding capacity (as described above), soil microcosms were constructed by adding 200 g of sieved (2 mm) and homogenized soil to one-half pint (approx. 237 mL) Ball jars (Alltrista Corp., Muncie, IN).

**Sampling of Soil Microcosms**

The time-dependent response of soil bacterial populations was assessed in microcosms amended with an aqueous succinate solution or sterile distilled water, and compared to unamended controls receiving no treatment. Microcosms were allowed to equilibrate at 25°C for seven days prior to treatment in order to lower moisture content and establish a baseline rate of respiration. At time of treatment (time = 0 hr), succinate-amended microcosms were adjusted to

30% of soil water holding capacity (% WHC) with an aqueous succinate solution (167 mM, pH 6.45) to 1 mg C per g soil (dry weight). Similarly, water-amended microcosms were adjusted to 30% WHC with sterile distilled water. All microcosms (including unamended controls) were homogenized immediately following treatment (0 hr) to normalize for treatment effects attributable to physical disturbance of the soil matrix. A destructive sampling scheme was employed where three replicate samples were removed at each time point (0 hr, 48 hr, 96 hr) for each treatment. Microcosms sampled at the 0 hr time point were processed within one hour following treatment.

At each destructive sampling time point ~15 g of soil was reserved for colony appearance curves and direct microscopic counts (see below) while the remainder was frozen in liquid nitrogen and stored at -80°C for subsequent extraction of nucleic acids. Soil moisture content was determined gravimetrically immediately prior to treatment and at each destructive sampling time point. All reported values were normalized to the dry mass of soil.

**Total Direct Cell Counts**

Bacterial cells were enumerated in soil samples using the protein-specific fluorescent dye DTAF (5-(4,6-dichlorotriazine-2-yl) amino fluorescein) (Sigma, St. Louis) following the method of Bloem (5). A total of three 15 mm diameter slide wells (10 randomly chosen fields per well) were averaged for each soil sample to obtain total cell counts. Putative eukaryotic cells including fungal hyphae, protozoa, etc., were not counted.

**Colony Appearance Curves for Soil Microcosms**

Colony appearance curves were determined for replicate treatments at 0 hr, 48 hr, and 96 hr sampling points. For each colony appearance curve, a 10 g portion of homogenized soil was suspended in 100 mL of sterile soil dilution buffer, mixed for 10 min on a magnetic stirrer, and a 1 mL portion of the suspension was serially diluted. Aliquots from the dilution series were plated on 1.5% agar medium containing a 100–fold dilution of Nutrient Broth (Difco Inc) and bacterial colonies were enumerated at periodic time intervals over 300 hours.

**Extraction of Nucleic Acids from Soils**

Total RNA was extracted from each microcosm for use in quantitative filter hybridization assays by following established methods (10, 29). Briefly, 10 g of soil was combined with 20 mL of soil homogenization buffer (SHB; 4M guanidium isothiocyanate, 200 mM sodium phosphate (pH 8.0), 25 mM sodium citrate, and 0.5% N-lauryl sarcosine), 20 g of 0.1 mm zirconia/silica beads (Biospec Products, Bartlesville, OK) and processed by mechanical disruption in a Mini Bead Beater (Biospec Products) for 2 min while cooled by an ice jacket. Particulate matter was removed by centrifugation and the pellet was washed with 10 ml of SHB. Supernatant fractions were pooled, precipitated with polyethylene glycol and sodium chloride, and recovered by centrifugation. Samples were washed twice with 70% ethanol and further purified by extraction with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1; pH 4.3). Humic acids were removed by column chromatography with hydroxyapatite (Bio-Rad Laboratories, Hercules, CA), desalted using Sephadex G-75 (Amersham

Pharmacia Biotech, Inc., Piscataway, NJ) spin columns, and recovered by sodium acetate/isopropyl alcohol precipitation. Purified RNA samples were spectrophotometrically quantified at wavelength of 260 nm. Absorption spectra from 220 – 320 nm were recorded to determine the quality of RNA samples and ensure removal of humic acids.

**Quantitative Hybridization of Ribosomal RNA**

Ribosomal RNA extracted from soil microcosms was quantified by hybridization on nylon membranes using 16S rRNA targeted probes as described previously (10, 39). Briefly, RNA samples (including controls) were denatured in gluteraldehyde, serially diluted to provide five different concentrations, blotted onto a positively charged nylon membane using a 96-well vacuum-blotter, and immobilized by UV crosslinking (120 mJ). Membranes were hybridized with $^{32}$P-labeled oligonucleotide probes targeting 16S rRNA representing the following phylogenetic groups (*probe name*): α-proteobacteria (Alf1b), β-proteobacteria (Bet42a), γ-proteobacteria (Gam42a), *Cytophaga-Flexibacter* (CF319), *Actinobacteria* (HGC69a), *Planctomyces* (Pla46ra; *unpublished data*), and *Eukarya* (Euk1195) (1). Group-specific rRNA hybridization signals were related to a universal probe binding to the *Bacteria*, *Archaea*, and *Eukarya* domains (Univ1390) (1). Membranes were hybridized at 45°C for ≥12 hr, washed at 45°C for 30 min, followed by a stringent wash at 45°C (Euk1195, Univ1390), 50°C (HGC69a), 55°C (Alf1b, CF319, Pla46ra), or 62°C (Bet42a, Gam42a). The amount of specifically bound probe remaining on membranes after stringent washing was quantified by transferring $^{32}$P β-particle emissions to Phosphor

Imaging Plates (Eastman Kodak, Rochester, NY), followed by quantitative imaging on a Molecular Dynamics Storm 860 fluorescent scanning system (Amersham Pharmacia Biotech, Inc.).

To control for non-specific hybridization, cross-hybridization, and differences in the radioactivity of probes, RNA extracted from pure cultures was included on each membrane. Control RNA included (*phylogenetic group represented*): '*Ketogluonogenium vulgarum*' (α-proteobacteria), *Nitrosomonas europaea* ATCC 25978 (β-proteobacteria), *Pseudomonas aeruginosa* ATCC 10145 (γ-proteobacteria), *Cytophaga johnsonae* ATCC 17061 (*Cytophaga-Flexibacter*), *Arthrobacter globiformis* ATCC 8010 (*Actinobacteria*), *Bacillis subtilis* ATCC 6051 (low mol%G+C Gram Positive), *Acidobacterium capsulatum* ATCC 51196 (*Acidobacteria*), *Planctomycetes limnophilus* ATCC 43296 (*Planctomycetes*), and *Saccharomyces cerevisiae* American Ale Yeast 1056 (Wyeast Labs, Inc.)(*Eukarya*). As a precaution against pipetting and handling errors, RNA samples from soil microcosms and controls were prepared at one time with the same pipettor, diluted with the same stock solution of gluteraldehyde, and immediately frozen at -20°C. Each 96-well blot should have therefore contained the same amount of sample and standards during hybridization.

The relative abundance of each group-specific probe (e.g. Alf1b) was calculated by determining the ratio of group-specific probe activity to Universal probe (Univ1390) activity while accounting for both positive and negative controls. The linearity of each RNA dilution series on membrane filters was

confirmed to ensure that signal saturation did not occur and that hybridization was not affected by contaminants such as humic acids. The amount of probe bound to soil RNA samples and control RNA were calculated by determining the mean signal of the five dots representing each sample dilution series. The activity of group-specific probes was determined by their ratio to positive controls while subtracting the ratio to negative controls on each blot to account for differences in probe-labeling efficiency, non-specific binding, and cross-hybridization (39).

Absolute rRNA abundance was calculated by multiplying the relative abundance of a microbial group against the total RNA (per g soil, weight). Normalizing relative abundance values to total RNA assumes that the universal probe binds to 100% of total RNA extracted from soil microcosms. Since rRNA is highly stable and survives harsh extraction procedures intact, we concluded this normalization procedure produced a reasonable estimation of community rRNA pools. These normalization procedures also assume that rRNA extraction efficiency is constant between replicate microcosms within and between treatments. Extraction efficiencies can vary considerably between soils of different composition due to binding of nucleic acids to soil matrix. Soil microcosms were established from a single homogenous soil sample, therefore the rRNA extraction procedure was assumed equally efficient among replicate microcosms.

**Statistical Analysis of rRNA Hybridization Data**

To correct for non-normal sample distributions, relative rRNA abundance values were transformed using the ArcSine function prior to statistical analysis (38). All tests of statistical significance were calculated, and are reported, from ArcSine transformed relative rRNA abundance values. A second concern with rRNA abundance values in a treatment was variation between populations that differed considerably in abundance. The coefficient of variance was calculated for each group within a treatment at each sampling time to compare variability between groups present in different absolute and relative rRNA abundances. Variability within a group should be independent of population size at a single time point. Mean-weighted variance analysis indicated that the coefficient of variance (CV) associated with group-specific hybridization signals was not affected by microbial group relative or absolute rRNA abundance within treatments (data not shown).

Correspondence analysis (CA) was used to explore treatment and time effects on the relative rRNA abundance of microbial populations in soil microcosms (34). CA is a weighted form of principal component analysis that is appropriate for frequency data such as relative abundance values obtained from rRNA dot blot hybridization (20). In CA, scores (inertia values) are computed for each row and column category in a contingency table, and plots of these scores show the relationships among the categories. The concept of inertia in CA is analogous to the concept of variance in principal component analysis. CA was implemented using the 'CORRESP' routine of the SAS software package (SAS

Institute Inc., Cary, NC) for rRNA hybridization data. Mean relative abundance values for each probe were calculated from replicate microcosms at each time point within treatments. Row categories were identified in CA as the nine time-treatment combinations (i.e. succinate-amended at 0hr, water-amended at 0 hr, unamended at 0hr, etc.) and column categories were the five probes with signals above the limit of detection (α- and β-classes of the *Proteobacteria*, *Actinobacteria, Eukarya,* and *Planctomycetes*).

The effects of treatment and time (and their interaction) resulting from changes in relative and absolute rRNA abundance within soil microcosms were examined using multiple analysis of variance (MANOVA) and Pillai's trace values. Pillai's trace test statistic is more robust to violations of test assumptions, and consequently more conservative (35). Tests of significance for changes in the absolute and relative rRNA abundance of specific microbial groups within and between treatments were calculated using analysis of variance (ANOVA). Post-hoc statistical analyses of treatment and time effects on relative and absolute rRNA group abundance were performed using Scheffé's mean separation test. All statistical tests were implemented with StatView version 5.0.1 (SAS Institute) and SAS/STAT version 8.1 (SAS Institute) on the Microsoft Windows 2000 (Redmond, WA) operating platform.

## RESULTS

### Response to Cultivation on Succinate Minimal Medium

A total of $2.8x10^6$ colony forming units (CFUs) per g soil were recovered on succinate minimal medium over 250 hr, representing approximately 0.35% of the total number of cells estimated by direct microscopic observation ($8.2 \times 10^8$ cells per g soil). Forty-three bacterial strains from early (≤62 hr) and late (≥198 hr) appearing time intervals were isolated in pure culture. Partial 16S rRNA sequence analysis demonstrated that cultivated bacteria include members from the *Actinobacteria*, α-, β-, and γ-classes of the *Proteobacteria* (Table 4.2). While early appearing colonies comprised isolates from all four of the former groups, late appearing isolates were restricted to the *Actinobacteria* and α-proteobacteria (Table 4.2). Early appearing bacteria from the *Actinobacteria* can be tentatively placed in the *Arthrobacter* genus, while late appearing *Actinobacteria* are primarily members of the *Mycobacterium* genus. Of the bacterial isolates characterized as α-proteobacteria, early appearing bacteria are primarily members of the *Rhizobiaceae* family, while late appearing bacteria are members of the *Bradyrhizobiaceae* and *Hyphomicrobiaceae* families (data not shown).

**TABLE 4.2.** Recovery of dominant microbial groups from soil and correlation between rRNA operon copy number and colony appearance time on succinate minimal medium.

| | Early Appearing Colonies[a] | | | | Late Appearing Colonies[a] | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Phylogenetic Group** | **%** | **n[b]** | **Mean rRNA #** | **95% CI** | **%** | **n[b]** | **Mean rRNA #** | **95% CI** | **rRNA Abundance[c]** |
| α-proteobacteria | 12 | 3 | 4.7 | 4.0 - 5.3 | 74 | 14 | 2.4 | 2.0 - 2.7 | 35.9% ± 2.0% |
| *Actinobacteria* | 23 | 6 | 5.0 | - | 26 | 5 | 1.8 | 1.4 - 2.2 | 13.6% ± 1.1% |
| *Planctomycetes* | - | - | - | - | - | - | - | - | 7.0% ± 0.5% |
| *Eukarya* | - | - | - | - | - | - | - | - | 6.3% ± 0.8% |
| β-proteobacteria | 23 | 6 | 4.8 | 4.2 - 5.3 | - | - | - | - | 2.6% ± 0.2% |
| γ-proteobacteria | 42 | 11 | 5.6 | 5.1 - 6.2 | - | - | - | - | < 0.5%[d] |
| *Cytophagales* | - | - | - | - | - | - | - | - | < 0.5%[d] |

[a] Values represent the percentage of all bacterial strains in each phylogenetic group isolated during that time interval. Mean rRNA operon copy number per genome were experimentally determined for strains in each phylogenetic group. Early appearing colonies, ≤62 hr; Late appearing colonies, ≥198 hours. 95% CI, 95% confidence interval for rRNA operon copy number.

[b] rRNA operon copy number values for individual strains are listed in Appendix B.

[c] range of rRNA hybridization values in all treatments and time points analyzed during soil microcosm experiments. Values are % relative rRNA abundance to Univ1390 probe, ± the standard error of the mean.

[d] below the level of detection (<0.5 %)

On average, early appearing bacteria possessed a greater number of rRNA operons per genome ($\bar{x}$ = 5.2, *n* = 26) than late appearing bacteria ($\bar{x}$ = 2.1, *n* = 17) (Table 4.2). Phylogenetic conservation of rRNA operon copy numbers for bacteria isolated on succinate minimal medium closely parallels values obtained for the same groups from the literature (Table 4.1 & 4.2). Closer examination of the *Actinobacteria* group reveals phylogenetically distinct subgroups of closely related bacteria with similar rRNA operon copy number. *Actinobacteria* is a phylogenetically diverse group primarily containing bacteria with greater than four rRNA operons per genome; however, the *Mycobacterium* genus (within the *Actinobacteria* group) contains bacteria limited to one or two rRNA operons per genome (Table 4.1). Data available from the literature for the α-proteobacteria indicates a group with primarily 1 to 3 rRNA operons, yet these experiments demonstrate that members of α-proteobacteria can contain up to five rRNA operons per genome (Table 4.2).

Quantitative rRNA hybridization (described in detail below) indicates that bacteria isolated on succinate minimal medium are from phylogenetic groups that account for approximately 56% of total rRNA present in soil microcosms. The most abundant group (relative and absolute abundance) quantified by rRNA hybridization, the α-proteobacteria, accounted for the majority of slow responding colonies appearing after 198 hr of cultivation (Table 4.2). Cultivation biases are apparent for the γ-proteobacteria, which accounted for 42.3% of early appearing isolates, but were below the detection limit of rRNA hybridization (<0.5%). The failure to isolate bacteria from phylogenetic groups representing approximately

44% of total rRNA that did not hybridize to any of the probes demonstrates additional biases imposed by cultivation.

**Global Community Structure in Soil Microcosms**

Soil RNA content indicated that succinate amendment had little positive effect on rRNA synthesis and identified potentially negative effects associated with water amendment alone. Total RNA extracted from soil microcosms ranged between 0.32 and 5.63 μg RNA•g soil$^{-1}$ and significantly increased within succinate-amended and unamended microcosms (Figure 4.1). No significant changes in soil RNA content occurred within water-amended microcosms over the course of the experiment. However, total RNA was significantly lower in water-amended microcosms relative to both the unamended and succinate-amended microcosms at 0 and 96 hours. A decrease in soil RNA may have resulted from cellular lysis and immobilization of RNA onto the soil matrix, however a similar decrease in soil RNA content was not observed in succinate-amended microcosms (Figure 4.1). Therefore, we were concerned that low RNA extraction yields, particularly in water-amended microcosms, were due to experimental error and not biological response to treatment.

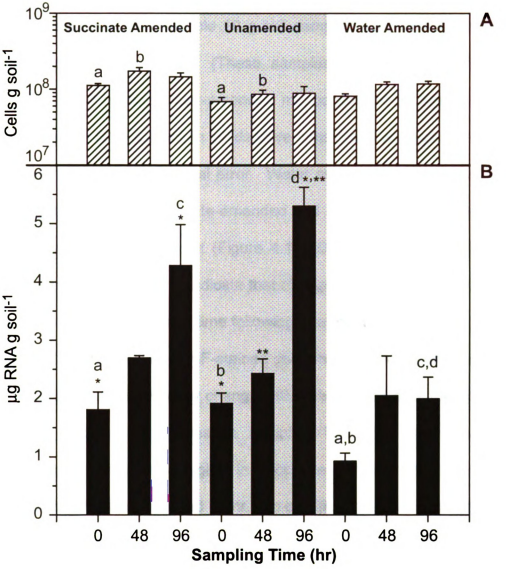


**FIGURE 4.1.** Changes in soil microcosm communities. (**A**) Total direct counts of DTAF-stained cells. Between treatment differences ($P < 0.05$) are noted with letters. (**B**) Total RNA quantified in soil microcosms. Filled bars indicate the average total RNA content from replicate microcosms ($n = 3$) within treatments at each sampling time. Between treatment differences in total RNA ($P < 0.1$) at each sampling time are denoted by letters; within treatments differences ($P < 0.05$) between sampling times are noted by asterisks. Error bars represent the standard error of the mean. Values are expressed per gram dry weight soil.

To control for methodological variability in RNA extraction yields, total RNA was extracted from multiple 10 g soil samples from replicate microcosms initially producing low yields. (These samples included succinate-amended microcosms at 0 hr, and water-amended microcosms at all three time points.) Consistently low RNA extraction yields were considered a result of low soil RNA content rather than experimental error. Water amendment decreased soil RNA content relative to both succinate-amended and unamended controls ($P < 0.12$) immediately following treatment (Figure 4.1). Differential effects of water and succinate amendment at 0 hr indicate that changes occurred in soil RNA content within the one hour processing time following treatment.

The total number of DTAF-stained cells enumerated by direct microscopic observation did not significantly change within treatments over the course of the experiment (Figure 4.1). Despite sampling less than one hour following treatment, cell counts were higher in succinate-amended microcosms than in unamended controls at 0 hr and 48 hr. No significant differences were observed between direct cell counts in water-amended and unamended microcosms. Based on direct cell counts, cellular RNA content varied between 11.4 fg and 59.9 fg RNA per cell within all treatments. Comparatively, bacteria characterized in Arctic marine sediments were estimated to contain ~3 fg RNA per cell (33). *E. coli* cellular rRNA content varies between 20 fg and 211 fg RNA per cell when growing between 24 and 100 min doubling times (8).

Microbial communities in soil microcosms were dominated by rRNA from the α-proteobacteria, followed by (in order of decreasing abundance) the *Actinobacteria, Planctomycetes, Eukarya*, and β-proteobacteria (Table 4.2). The γ-proteobacteria and the *Cytophagales* were below the limit of detection (approximately 0.5% of the total RNA recovered) in all samples analyzed before and after treatment. The five probes producing quantifiable signals accounted for an average of 65.4 ± 4.7% S.E.M. of total rRNA detected by the universal probe, of which 59 ± 3.9% S.E.M. is bacterial rRNA (universal signal less *Eukarya* signal). Groups not interrogated by rRNA hybridization in soil microcosms, but commonly found in soil by nucleic-acid based methodology (9), include: *Acidobacteria*, *Verrucomicrobia*, *Firmicutes*, *Cytophagales*, and the *Clostridium*. Assuming rRNA hybridization accurately reflects the *in situ* microbial community, the total number of cells detectable by microscopic direct counts prior to amendment ($8.1\times10^7$) included: $2.9\times10^7$ α-proteobacteria, $1.1\times10^7$ *Actinobacteria,* $5.7\times10^6$ *Planctomycetes,* and $2.1\times10^6$ β-proteobacteria cells per g soil. Accordingly, the γ-proteobacteria and the *Cytophagales* represented less than the rRNA detection limit of approximately $4.1\times10^5$ cells per g soil.

**Effects of Treatment on the Cultivable Microbial Community**

Colony appearance curves were used to characterize microbial communities in soil microcosms based on the percentage of cultivable bacteria appearing during different time intervals following plating (19, 21). Immediately following amendment (t = 0 hr), little difference was apparent in the total number of cultivable bacteria between treatments (Figure 4.2). At 48 hr following amendment with succinate, the total number of cultivable colonies increased 11-fold (Figure 4.2). Over 66% of the increase in cultivable bacteria in succinate-amended microcosms between 0 – 48 hr is attributable to colonies appearing less than 66.5 hr following plating. Colonies appearing less than 66.5 hr after plating were previously identified as bacteria with many rRNA operons ($\bar{x}$ = 5.2) per genome (Table 4.2). Colonies appearing in water-amended and unamended control microcosms accounted for only 33% and 29% of total cultivable bacteria, respectively, during the same time interval. Water amendment resulted in 2.1-fold increase of cultivable bacteria between 0 hr and 48 hr. The positive slope of the curves formed by the appearance of bacterial colonies over time (Figure 4.2) indicates that increases in cultivable bacteria from water-amended microcosms are attributable to colonies appearing up to 150 hrs following plating from both 48 hr and 96 hr soil samples. Cultivable bacteria increased 1.2-fold within unamended microcosms over the course of the experiment (0 – 96 hr).

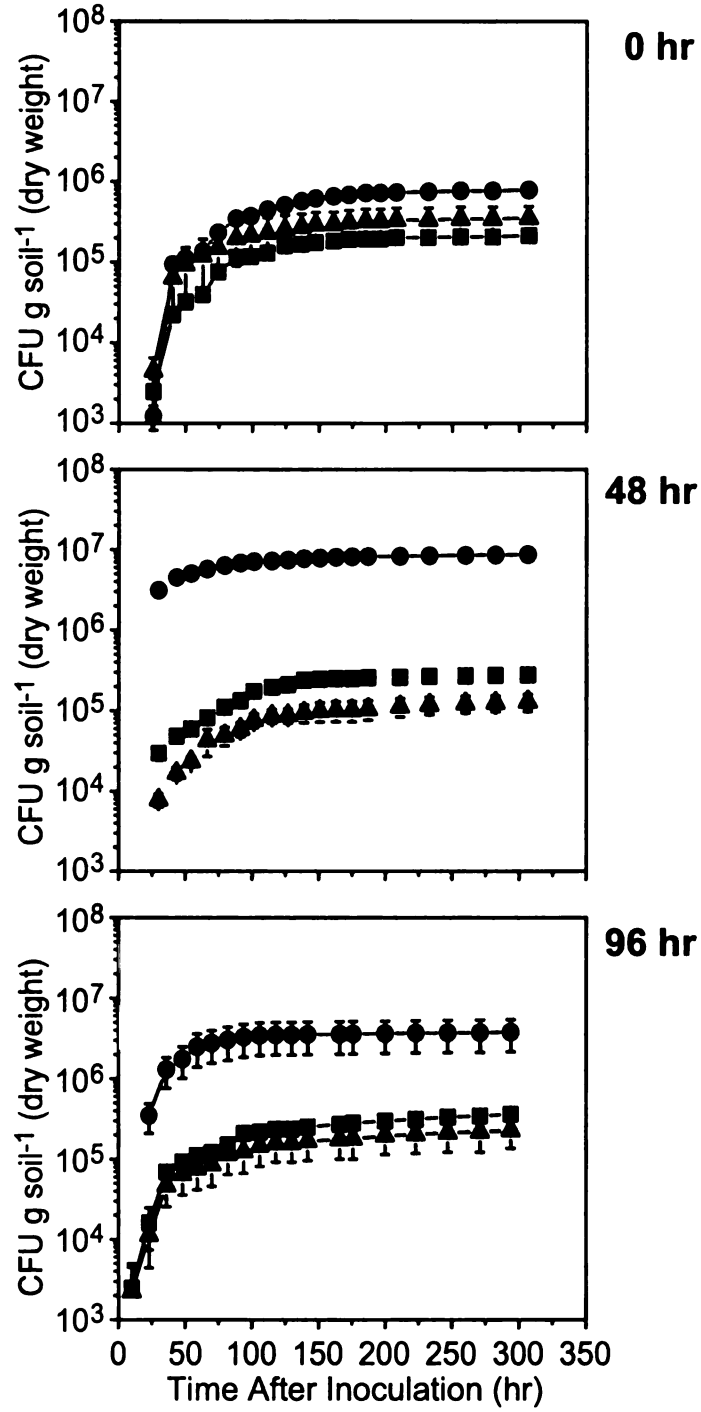


**FIGURE 4.2.** Cultivation response of soil microbial populations to succinate amendment in laboratory microcosms. Colony response curves from microcosms destructively sampled at 0 hr, 48 hr, and 96 hr following amendment. Succinate-amended (circles), water-amended (squares), and unamended microcosms (triangles). Bars represent the standard error of the mean.

**Effects of Treatment on Bacterial rRNA Synthesis**

Ecologists often relate the number of biological species to the aggregate total to describe the influence of populations on community structure. Relative abundance measurements are not sensitive to changes in numerically small populations in a dynamically changing community – a comparatively small population can double in number (in this case rRNA content) without a significant increase in relative abundance. The soil microbial community in our microcosms is composed of microbial populations largely disparate in size, which may preclude detection of changes in the relative abundance of populations in low abundance (Table 4.2). Absolute population size circumvents many difficulties associated with proportional species relationships, but fails to indicate the contribution of an individual species to overall community structure. We were interested in whether the colony appearance time of bacteria with many rRNA operons corresponded to an increased capacity for rRNA synthesis. An increased capacity for rRNA synthesis would permit immediate and rapid increases in rRNA abundance of bacterial groups possessing many rRNA operons. Since we were also interested in the contribution of bacterial groups to community structure, both relative (group-specific probe signal expressed as a percentage of the universal probe signal) and absolute (ng rRNA•g soil$^{-1}$, dry weight) rRNA abundance were analyzed.

Significant effects due to treatment, time, and the interaction of treatment and time were demonstrated by MANOVA of both relative and absolute rRNA abundance values (Table 4.3). ANOVA of both absolute and relative rRNA abundance indicated significant global effects ($P < 0.05$) due to treatment for all microbial groups, except for *Eukarya* absolute rRNA abundance. Significant global effects on absolute rRNA abundance due to time (within treatments) were observed for all microbial groups ($P < 0.05$), while global time effects for relative rRNA abundance measurements were limited to the β-proteobacteria and *Actinobacteria* ($P < 0.001$).

**TABLE 4.3.** Global effects of treatment and time on microbial community rRNA assessed with MANOVA.

**A. Relative rRNA Abundance**

| Effect | df | Pillai's Trace | F value[a] |
|---|---|---|---|
| Treatment | 10,30 | 1.84 | 33.6*** |
| Time | 10,30 | 1.12 | 3.8* |
| Treatment x Time | 20,68 | 1.99 | 3.4*** |

**B. Absolute rRNA Abundance**

| Effect | df | Pillai's Trace | F value[a] |
|---|---|---|---|
| Treatment | 10,30 | 1.79 | 25.3*** |
| Time | 10,30 | 1.27 | 5.2** |
| Treatment x Time | 20,68 | 2.11 | 3.8*** |

[a] (*, $P < 0.01$; **, $P < 0.001$, ***, $P < 0.0001$)

Histograms of absolute and relative rRNA abundance values indicate appreciable changes in the soil microbial community primarily due to the α-proteobacteria and *Actinobacteria* (Figure 4.3). A nearly 3-fold increase (9.5 to 24.3%) of *Actinobacteria* relative rRNA abundance between 0 to 48 hr due to succinate amendment was the largest response documented among all microcosms. Other appreciable changes within treatments include an increase of α-proteobacteria relative rRNA abundance (34.9 to 42.1%) between 0 and 48 hr in unamended microcosms, and an increase in the β-proteobacteria (2.2 to 5.1 %) in unamended microcosms between 0 and 96 hr (Figure 4.3). No appreciable increase in relative rRNA abundance of any microbial group was observed in water-amended microcosms, however the α-proteobacteria were considerably lower compared to other treatments. While the relative rRNA abundances of microbial groups were generally stable over the course of the experiment, the absolute rRNA abundance of all microbial groups increased from 0 to 96 hr in all three treatments indicating metabolic activity independent of amendment (Figure 4.3).

**FIGURE 4.3.** Response of soil microbial populations to succinate and water amendment in laboratory microcosms relative to an unamended control. **Top panel:** percent relative abundance (to Universal probe) of microbial groups at 0 hr, 48 hr, and 96 hr following treatment. **Bottom Panel:** absolute rRNA abundance (ng rRNA•g soil$^{-1}$).

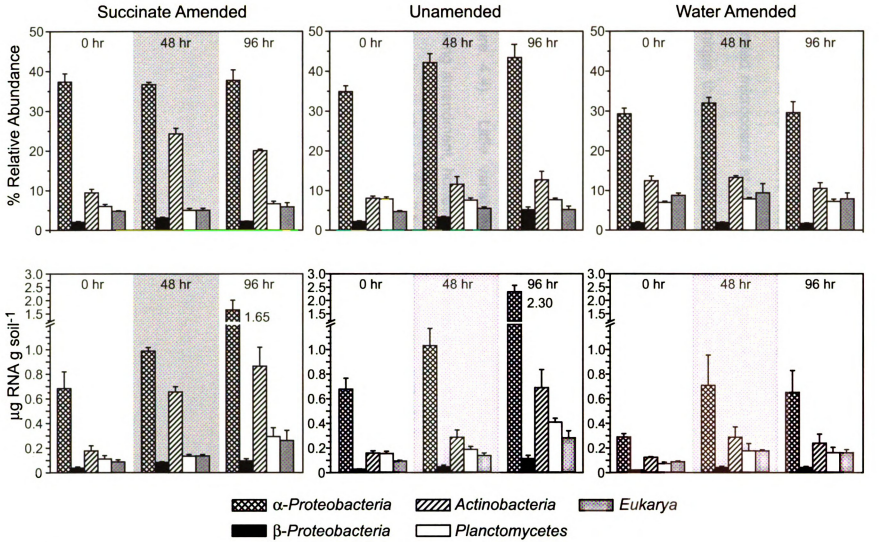
Succinate Amended
Unamended
Water Amended
0 hr
48 hr
96 hr
% Relative Abundance
µg RNA g soil-1
1.65
2.30
α-Proteobacteria
β-Proteobacteria
Actinobacteria
Planctomycetes
Eukarya

**FIGURE 4.3.**

Exploratory data analysis with correspondence analysis (CA) provided evidence that microbial community structure was largely associated with increases in the relative rRNA abundance of *Actinobacteria* in succinate-amended microcosms at 48 and 96 hr (Figure 4.4). (CA is a data visualization technique that relates community variability with different variables – in this implementation: time, treatment, and group-specific probes.) Changes in community structure are also apparent in water-amended microcosms that are potentially associated with variability in the relative abundance of the *Eukarya*. Intriguingly, the most abundant group of bacteria in soil microcosms, the α-proteobacteria, contributed little to changes in microbial community dynamics (Figure 4.4). Little variability was anticipated in microcosms immediately following amendment, however CA indicates appreciable variability in microbial community structure at the 0 hr time point (Figure 4.4). Differences observed in water-amended microcosms at 0 hr persisted throughout experimentation, suggesting that water amendment had an immediate and lasting impact on microbial community structure.

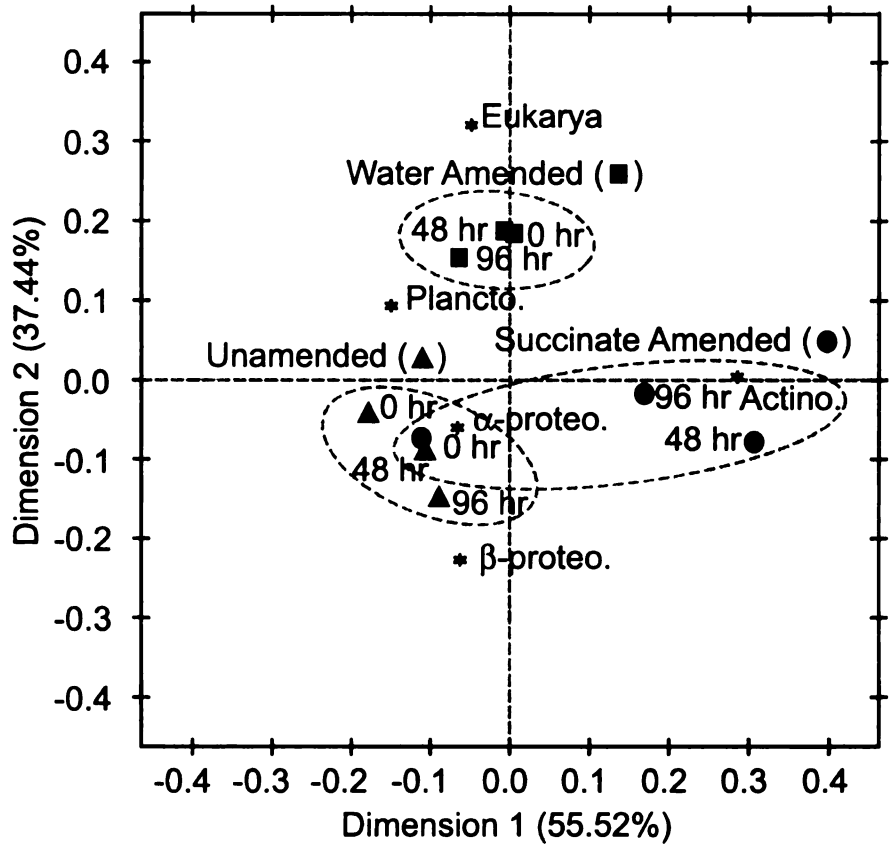


**FIGURE 4.4.** Soil microcosm relative rRNA abundance data assessed using correspondence analysis. Contributions of microbial groups to community variability are denoted by asterisks; variability associated with sampling times within treatments are identified by circles (succinate-amended), triangles (unamended), and squares (water-amended). Points enclosed by ellipses are to aid in visualization of treatment level variability over all three sampling times.

**Interpretation of Microbial Community Dynamics using Absolute & Relative rRNA Abundance Measurements**

Despite increases in rRNA content of all microbial groups from all treatments, only increases in total rRNA from the *Actinobacteria* and β-proteobacteria resulted in significant increases in relative rRNA abundance. The relative abundance of *Actinobacteria* rRNA in succinate-amended microcosms at 48 hr (24.3%) is significantly greater compared to water-amended (13.3%, $P < 0.05$) and unamended microcosms (11.6%, $P < 0.05$), indicating a response solely attributable to succinate amendment (Table 4.4). β-proteobacteria rRNA also increased in succinate-amended microcosms (3.1%) relative to water-amended microcosms (1.9%), at 48 hr ($P < 0.05$). Increases in the relative and absolute abundance of *Actinobacteria* and β-proteobacteria rRNA between 0 and 96 hr primarily resulted from rapid increases in total rRNA between 0 and 48 hr and not a continual increase over the course of the experiment (Table 4.4). The rapid increase in rRNA of *Actinobacteria* and β-proteobacteria is congruent with observations based on colony appearance time on succinate minimal media (Table 4.2), and with the increased abundance of early colony-forming bacteria in soil microcosms 48 hr following succinate amendment (Figure 4.2).

The synthesis of rRNA within treatments generally produced little change in the relative rRNA abundance of microbial groups in water-amended and unamended microcosms. rRNA content significantly increased ($P < 0.05$) in nearly all populations in unamended control microcosms, yet produced few

significant changes in relative rRNA abundance (Table 4.4). Despite sampling within one hour following treatment, the relative abundance of *Eukarya* rRNA is significantly greater ($P < 0.05$) in water-amended microcosms (9%) than in unamended (4.7%) and succinate-amended microcosms (4.8%) at 0 hr (Table 4.4). However, the increase in relative rRNA abundance of the *Eukarya* was accompanied by a decrease in total rRNA, indicating that increases in relative rRNA abundance likely resulted from eukaryal resistance to cellular lysis. Water amendment also negatively affected the most dominant microbial group in soil, the α-proteobacteria, which decreased in absolute rRNA abundance by 40%, relative to unamended microcosms at 0 hr ($P < 0.06$).

**TABLE 4.4.** Percent change in microbial group rRNA abundance assessed using absolute (ng rRNA•g soil$^{-1}$) and relative (% to Univ1390) rRNA abundance.[a]

**A. Between Sampling Time**

| Probe | Treatment | 0 / 48 hr | 48 / 96 hr | 0 / 96 hr |
|---|---|---|---|---|
| α-*Proteo.* | S | 44 (-2) | 67 (3) | 141 (1) |
| β-*Proteo.* | S | 120 **(52)** | 15 **(-28)** | **152** (9) |
| *Actino.* | S | **271 (156)** | 32 (-17) | **389 (112)** |
| *Plancto.* | S | 20 (-17) | 11 (34) | 33 (11) |
| *Euk.* | S | 54 ( 5) | 93 (17) | 198 (23) |
| α-*Proteo.* | U | 53 (21) | **124** (3) | **242** (24) |
| β-*Proteo.* | U | 77 (52) | 154 (56) | **350 (137)** |
| *Actino.* | U | 82 (43) | 144 (10) | **343** (57) |
| *Plancto.* | U | 22 (96) | **121** (1) | **169** (-3) |
| *Euk.* | U | 50 (17) | 107 (-5) | **211** (10) |
| α-*Proteo.* | W | 147 (9) | -8 (-7) | 127 (1) |
| β-*Proteo.* | W | 123 (2) | -5 (-10) | 113 (-8) |
| *Actino.* | W | 137 (6) | -7 (-21) | 96 (-16) |
| *Plancto.* | W | 149 (13) | -8 (-8) | 128 (3) |
| *Euk.* | W | **204** (7) | -9 (-16) | **86** (-10) |

**B. Between Treatment**

| Probe | Time (hr) | S / U | S / W | W / U |
|---|---|---|---|---|
| α-*Proteo.* | 0 | 2 (7) | 156 **(28)** | -60 (-16) |
| β-*Proteo.* | 0 | 56 (-7) | 132 (10) | -33 (-15) |
| *Actino.* | 0 | 14 (17) | 57 (-24) | -27 **(54)** |
| *Plancto.* | 0 | -26 (-24) | 71 (-14) | -57 (-11) |
| *Euk.* | 0 | -2 (2) | 10 **(-45)** | -11 **(87)** |
| α-*Proteo.* | 48 | -4 (-13) | 50 (15) | -36 (-34) |
| β-*Proteo.* | 48 | 94 (-16) | **128 (64)** | -15 (-43) |
| *Actino.* | 48 | **133 (110)** | **146 (83)** | -6 (15) |
| *Plancto.* | 48 | -27 **(-34)** | -18 **(-47)** | -11 (4) |
| *Euk.* | 48 | 1 (-8) | -17 (-47) | 21 (72) |
| α-*Proteo.* | 96 | -28 (-13) | 172 (28) | **-74 (-32)** |
| β-*Proteo.* | 96 | -13 **(-57)** | 175 (30) | -68 **(-67)** |
| *Actino.* | 96 | 26 (58) | **293 (91)** | -68 (-17) |
| *Plancto.* | 96 | -63 (-13) | 0 (-8) | **-63** (-6) |
| *Euk.* | 96 | -16 (13) | 76 (-25) | -47 (52) |

[a] percent change absolute and relative rRNA abundance (in parentheses) for microbial groups between sampling times (0, 48, 96 hr) and between treatments (S, succinated amended; U, unamended; W, water amended).

[b] Values in bold type indicate significant differences at the 95% confidence level (Scheffé's mean separation test)

## DISCUSSION

Quantative hybridization of rRNA provided an opportunity to independently monitor the metabolic activity of phylogenetically related groups of bacteria in complex microbial community assemblages. By combining the taxonomic resolution of rRNA-hybridization with direct cell counts and cultivation-based characterization, we were able to observe striking microbial community dynamics resulting from amendment of soil microcosms with succinate, water, and even in unamended controls. The theoretical basis of experimentation was that bacterial populations with many rRNA operons per genome possess an increased capacity for rRNA synthesis, which permits rapid increases in rRNA abundance and population size (Chapter 3).

Cultivation of soil bacteria on media containing succinate as the sole source of carbon and energy produced a taxonomically diverse collection of bacterial isolates with a wide range of rRNA operons per genome. The colony appearance times of these bacterial populations were directly related with rRNA operon copy number (Table 4.2). Despite the selective effects of growth on a single limiting carbon and energy source, cultivated bacteria represented a large proportion of the extant taxonomic diversity in soil. Perhaps most notable was the preponderance of late appearing bacteria with 1 to 3 rRNA operons classified as $\alpha$-proteobacteria. The $\alpha$-proteobacteria was the most abundant microbial group identified by rRNA hybridization in soil microcosms. Conversely, the $\gamma$-proteobacteria accounted for <0.5% of the *in situ* soil community rRNA, yet represented a large percentage of cultivable bacteria from soil (Table 4.2).

Increased rRNA synthesis capacity may confer little competitive advantage when nutrients are infrequently available in abundance, as in the carbon-limited environment of agriculturally managed soils.

Effects of succinate amendment in soil microcosms were apparent in the abundance of early and late colony forming bacteria that appeared following amendment. Succinate amendment increased the total number cultivable bacteria by an order of magnitude (11-fold) over water-amended microcosms after 96 hours (Figure 4.2). The increase in cultivable bacteria in succinate-amended microcosms was attributed to early appearing bacteria with many rRNA operons (≥4) per genome. Soil organic matter made available through water amendment was insufficient to support significant growth beyond unamended control microcosms during the course of experimentation. Organic content of soils used for experimentation is approximately 1% (by weight); therefore, soil microcosms (200 g total) contained approximately 2 g of total organic matter (32). Much of the soil organic matter was recalcitrant to rapid (within 96 hr) mineralization by microorganisms considering the substantial increase in cultivable bacteria within 48 hr of receiving only 1 mg C•g soil$^{-1}$ of a readily utilizable carbon source. However, soil organic material clearly supported metabolic activity (discussed below) in unamended microcosms (Figure 4.1).

Initial observation of total RNA extracted from soil microcosms, particularly increases in unamended control microcosms, led us to suspect that RNA extraction procedures were methodologically inaccurate. Repeated and consistent RNA extractions from replicate microcosms provided strong evidence

that the initial observations were, in fact, an accurate reflection of differential responses to amendment. The assumption prior to experimentation was that rRNA in both succinate- and water-amended microcosms would increase above unamended controls. Rather, total RNA in unamended microcosms increased at levels equivalent to succinate-amended microcosms. In addition, total RNA immediately decreased following water amendment (at 0 hr) and did not recover to levels comparable to unamended microcosms (Figure 4.1).

The immediate decrease of total RNA in water-amended microcosms may be attributable to cellular lysis resulting from the 9% change in moisture content. Biomass released from dried-and-rewetted soils is proportional to the magnitude of change in water potential, presumably a result of carbon made available from cellular lysis (13, 28, 42). RNA released from cells during water amendment is unlikely recovered from soil due to binding of free RNA with the soil matrix (17). Conversely, a decrease in total RNA at 0 hr was not observed in succinate-amended microcosms that were also wetted to 30% of the soil water holding capacity (Figure 4.1). An increased abundance of cultivable bacteria at 0 hr in succinate-amended microcosms (Figure 4.2) indicates that rRNA synthesis may have occurred during the one-hour processing time following treatment. Apparent effects of cellular lysis inferred from rRNA content in succinate-amended microcosms may have been ameliorated by immediate increases in rRNA synthesis within the one-hour processing time following treatment.

Total direct cell counts do not prima facie support the theory that changes in water potential caused cellular lysis. While cell counts were greater in succinate-amended microcosms than in unamended microcosms at 0 hr and 48 hr, no other significant differences were observed within or between microcosms over the course of the experiment despite large differences in extractable RNA (Figure 4.1). Two factors may explain these lack of differences: 1) direct cell counts were performed with a protein-based stain (DTAF) that does not distinguish between live and dead cells, and 2) only a small fraction of respiration is coupled with growth. First, the inability to distinguish between live and dead cells with a protein-based dye only permits the observation of increases in total cell counts during short-term experiments if dead cells remain primarily intact. Cell counts did not decrease within any of the soil microcosms. Secondly, if only a small fraction of metabolic activity is attributable to cellular division, changes in cell counts may be difficult to detect. Blagodatsky et al. (2000) estimated that only 0.5% to 0.8% of soil microbial respiration results from actively dividing bacterial populations (4). Changes in such a small percentage of the total soil microbial community would be difficult to detect given variability in direct cell counts from soil. The relationship between growth rate and cellular RNA content permits the direct measure of microbial metabolic activity, which provides considerably greater sensitivity than direct microscopic counts.

Microbial communities in soil microcosms were clearly dominated by α-proteobacteria. Changes observed in α-proteobacteria rRNA were limited to increases in absolute rRNA abundance that did not result in increases in relative

rRNA abundance (Figure 4.3). Significant changes in α-proteobacteria absolute rRNA abundance occurred solely in unamended control microcosms and not in response to succinate amendment (Table 4.4). Succinate-amendment also had little effect on the cultivation of the α-proteobacteria (Figure 4.2), which were identified by cultivation as primarily late appearing colonies with 1 to 3 rRNA operons per genome (Table 4.2). The failure of the α-proteobacteria to respond to succinate amendment and their dominance of the soil microbial community indicates that a lower rRNA synthesis capacity may be advantageous in the carbon-limited environment of soil. One indication of the success of this ecological strategy is the dominance of the α-proteobacteria in nutrient-limiting soils of diverse composition and origin, including KBS-LTER soils (9, 23, 24, 30, 44).

Metabolic activity inferred from increased rRNA content was not limited to the α-proteobacteria in unamended microcosms. Over the 96 hr experiment, all microbial groups in unamended control microcosms demonstrated >150% increases in the amount of rRNA per g soil (Table 4.4). Absolute rRNA abundance increased equivalently among microbial groups in unamended microcosms such that only the β-proteobacteria increased in relative rRNA abundance. By far the greatest effects were observed in succinate-amended microcosms (Figure 4.3). Rapid rRNA synthesis in the *Actinobacteria* and β-proteobacteria increased the relative abundance of these groups at 48 hr, but remained constant or actually decreased between 48 – 96 hr (Table 4.4). While *Actinobacteria* and β-proteobacteria were able to rapidly utilize resources made

available through succinate amendment, the degree of competitive success associated with this capability likely depends on the frequency and magnitude of fluctuations in resource availability. The low relative abundance of *Actinobacteria* (9.5%) and β-proteobacteria (2%) in the soil community prior to amendment suggests potential long-term costs associated with maintenance of an increased capacity for rRNA synthesis in soil where resources are infrequently abundant.

Measurement of both absolute and relative rRNA abundance provided a dynamic view of microbial community activity in soil microcosms. Analysis of percent changes (increases and decreases) in absolute and relative rRNA abundance provided valuable insight into how underlying increases in metabolic activity are responsible for changes in the relative abundance of microbial populations (Table 4.4). Changes in the absolute rRNA abundance of most microbial groups did not vary proportionally to relative rRNA abundance. What appeared to be extremely stable microbial communities in unamended control microcosms based on relative abundance measurements, were revealed as dynamic communities capable of rRNA synthesis with limited resources and low water availability. Additionally, increases in both relative and absolute rRNA abundance provided a means to quantify the contribution of microbial populations to community structure when a large percentage of the community was not surveyed.

All microbial groups interrogated by rRNA hybridization demonstrated increases in rRNA synthesis following treatment. Studies of agriculturally managed soils in our laboratory using quantitative rRNA hybridization indicate

that microbial communities are remarkably stable over periods as long as decades (11). Experiments described above indicate that significant short-term dynamics underlie this long-term stability. A change in soil moisture content of 9% reduced the rRNA content of soil by >50%, while total RNA more than doubled in unamended microcosms (Figure 4.1). Total RNA in water-amended microcosms only recovered to pre-treatment values, reflecting the paucity of readily utilizable nutrients in soil.

Phylogenetically related groups of bacteria with many rRNA operons were predicted to possess an increased capacity for rRNA synthesis, conferring the ability to rapidly increase rRNA abundance and growth immediately following succinate amendment. Colony appearance time and increases in rRNA abundance were directly related to rRNA operon copy number. Bacterial groups with both many and few rRNA operons demonstrated significant increases in rRNA abundance over the 96 hr experiment, but the magnitude of response was much greater for populations with many rRNA operons between 0 hr and 48 hr. Only increases in rRNA synthesis by populations with many rRNA operons produced changes in the cultivable bacterial community.

Relative fitness in the carbon-limited environment of soil may be higher for organisms that can survive long periods of resource scarcity rather than rapidly increasing population size. The dominance of the soil microbial community by bacteria with few rRNA operons may reflect low metabolic costs associated with the maintenance of a lower capacity for rRNA synthesis. Rapid increases in the cultivability and rRNA abundance of bacterial populations with many rRNA

operons provide evidence that relative fitness of this group is determined by the availability of abundant resources. The low abundance of microbial populations with many rRNA operons in the soil may indicate that a metabolic capacity for rapid exploitation of nutrients is an ecological strategy with lower competitive success in the carbon-depleted environment of agriculturally managed soils. Bacteria with either many or few rRNA operons are capable of utilizing scarce resources as evidenced in unamended microcosms, however the high abundance of bacteria with few rRNA operons indicates a trade-off associated with maintaining high rRNA synthesis capacity during long-term growth in the carbon-limiting environment of soil.

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# CHAPTER 5

## WHY GROW SLOWLY? TEST FOR AN ECOLOGICAL TRADE-OFF BETWEEN NUMERICAL CELL YIELD AND MAXIMAL GROWTH RATE IN BACTERIA WITH DIFFERENT NUMBERS OF RIBOSOMAL RNA OPERONS PER GENOME.

## INTRODUCTION

Ecological strategies describe the life-history traits of species that provide a competitive fitness advantage for shared limiting resources. One prominent feature of ecological strategies is trade-offs in fitness for success in one environment versus another. The theoretical basis of trade-offs is the inability of an organism to simultaneously maximize all phenotypic components of fitness with a limiting resource pool (23). Trade-offs in plants are often associated with resource allocation to competition versus reproduction ("root or shoot")(7), or fecundity versus longevity of offspring. Attempts to identify ecological strategies in bacteria have largely focused on competition for abundant versus scarce resources (12, 13, 22, 36, 37). The historic basis for this dichotomy can likely be traced to Winogradski's characterization of soil bacteria as 'zymogenous' (rapid growth on readily utilizable substrates) and 'autochthonous' (slow growth on recalcitrant organics) (in ref. 2). These classifications have been similarly referred to as 'copiotrophic' or 'oligotrophic', (25) and 'r-' or 'K-' selected bacteria, respectively (1).

Based on observations such as Winogradski's, it is generally perceived that bacteria are unable to maximize fitness for growth in environments where resources are continually limiting or are periodically abundant. Demographic parameters of bacterial growth modeled by Monod-type kinetics classify copiotrophic bacteria as possessing high maximal growth rates ($\mu_{max}$) and low substrate affinity (high $K_s$, substrate concentration at $0.5 \cdot \mu_{max}$), and oligotrophic bacteria as low $\mu_{max}$ and high substrate affinity (low $K_s$). Experimental studies of

bacterial resource competition have generally not supported a trade-off in competitive ability for the utilization of abundant versus scarce resources (17, 18, 35, 37, 38). Long-term experimental studies with *Escherichia coli* indicate that evolutionary adaptation to a seasonal regime ("feast-and-famine") does not negatively affect fitness in an environment supporting constant low growth (35). In laboratory evolution experiments with bacteria recently isolated from soil, Velicer & Lenski (1999) concluded that adaptation to resource abundance or scarcity does not sacrifice fitness in the alternative regime – a fitness trade-off does not exist between $\mu_{max}$ and $K_s$ (37). The inability to demonstrate a trade-off in competitiveness for resource abundance and scarcity does not preclude the possibility that trade-offs exist in other bacterial life-history traits.

Resource competition theory assumes growth is limited by a single resource, and the organism reducing this resource to the lowest level ($R^*$ or $J$) will drive all other species to extinction, in a spatially unstructured habitat (9, 32). The validity of this theory has been demonstrated in both microbial (33) and plant systems (34). Obviously, coexistence is the rule rather than the exception in biological communities. What ecosystem parameters, then, support the extant diversity of microorganisms and how do ecosystem processes shape ecological strategies for survival and persistence? One clear difference between experimental and natural resource competition is the presence of spatial subdivision in the environment. In plant species, spatial subdivision permits the coexistence of an infinite number of species competing for a shared limiting resource, given requisite trade-offs in competitive ability, dispersal, and longevity

(31). Whether analogous trade-offs of dispersal, competitive ability, and longevity can support coexistence of bacteria competing for a single limiting resource in a spatially subdivided habitat is unknown. One can infer that microbial diversity in soil is supported by the enormous spatial heterogeneity of the soil matrix, but other factors clearly affect the distribution of microbial populations. Vasi et al. (35) demonstrated that selection for maximal growth rates in evolved populations of *E. coli* were accompanied by a decrease in numerical cell yield, due to increased cell size. A trade-off between maximal growth rate and numerical cell yield could reduce the fitness of rapidly growing microbial populations in a spatially structured environment, such as soil, by decreasing dispersal rates and increasing the probability of extinction due to chance events (35). Additionally, metabolic costs associated with the synthesis of larger cells could effectively decrease fitness in an environment with constant low resource availability.

Characterization of soil microbial communities in our laboratory has demonstrated that bacterial populations with few (≤3) rRNA operons comprise the largest fraction of the soil microbial community (Chapter 4, and ref. 4). rRNA operon copy number is a genetic determinant of central metabolic capacity that sets an upper limit on the rate of ribosome biosynthesis (Chapter 1). A direct correlation exists between rRNA operon copy number and the time required to form visible colonies on solid agar media. Bacteria with many ($\bar{x}$ = 5.5) rRNA operons form visible colonies more rapidly than late appearing colonies with few ($\bar{x}$ = 1.4) rRNA operons (Chapter 3). Slow colony appearance

times by bacteria with few rRNA operons reflects a lower capacity for rRNA synthesis (Chapter 4). The dominance of soil microbial communities by bacteria with few rRNA operons suggests that a low metabolic capacity is competitively advantageous in the soil environment. These observations, and those discussed above, led us to question whether a trade-off exists between numerical cell yield and maximal growth rate. Perhaps the dominance of bacteria with few rRNA operons in the carbon-limited soil environment is explained, in part, by their ability to efficiently convert limiting resources into progeny. Metabolic costs associated with the maintenance of increased rRNA synthesis capacity may decrease the efficiency with which bacteria with many rRNA operons can convert resources into progeny, an effect that would be especially evident at low resource concentrations. These metabolic costs may include both transcriptional regulation of a greater number of rRNA operons and constitutive expression of rRNA in excess translation requirements at slow growth rates.

The hypothesis of a trade-off between numerical cell yield and maximal growth rate was tested with soil bacteria selected based on colony appearance time and phylogeny. Twelve strains were selected based on colony appearance time (6 strains appearing ≤62 hr and 6 strains appearing ≥198 hr following plating) and grown on a nutritionally complex medium over a 5000-fold concentration range. Growth rates were determined at substrate saturating conditions during periods of exponential growth. Results were analyzed by using both traditional comparative methods and phylogenetically independent contrasts that correct for statistical biases resulting from the non-independence of

evolutionarily related organisms (6). This research was not an attempt to provide a mechanistic explanation for a trade-off in numerical cell yield, but to first ascertain whether such a trade-off exists. Bacterial fitness is undoubtedly influenced by many biological parameters, each of which contributes to competitiveness under unique sets of environmental selective pressures.

## MATERIALS & METHODS

### Bacterial Strains

Bacterial strains used for numerical yield studies were isolated from soil under intensive agricultural management collected at the Kellogg Biological Station Long Term Ecological Research (KBS-LTER) site (Hickory Corners, Michigan) on October 9, 1998. Bacterial strains were isolated based on colony appearance time on agar medium containing basal salts (Appendix A) and 5 mM succinate as a sole source of carbon and energy (Chapter 4). Briefly, pure cultures of bacteria were obtained by transferring a single isolated colony on solid agar medium a minimum of six times. The purity of cultures was verified by colony morphology and light microscopy, and freezer stocks (20% glycerol) were prepared and stored at -80°C. Amplified ribosomal DNA restriction analysis was used to identify genetically distinct bacterial strains (16), which were further characterized by phylogenetic analysis of partial 16S rDNA sequences (Chapter 3). Forty-three bacterial strains, in total, from early (≤62 hr) and late (≥198 hr) appearing time intervals were isolated in pure culture and classified based on rRNA operon copy number (see Materials & Methods in Chapter 3). A subset of twelve strains was

selected that maximized phylogenetic diversity of both early appearing bacteria ($n$ = 6) with many (4 - 6) rRNA operons and late appearing bacteria ($n$ = 6) with few (2 - 3) rRNA operons.

**Phylogenetic Analysis of 16S rRNA Genes**

Near full-length 16S rRNA sequences were obtained (for the subset of 12 strains) from 16S rRNA gene products amplified from genomic DNA with primers corresponding to positions 5 - 27 and 1519 - 1540 of the *Escherichia coli* 16S rRNA gene using established methods (ABI 16S rRNA MicroSeq Gene Kit; PE Applied Biosystems Inc., Foster City, CA). Sequences from 16S rRNA PCR amplicons were obtained with an overlapping set of 12 primers (ABI 16S rRNA MicroSeq Gene Kit; PE Applied Biosystems) on an ABI 373XL DNA fluorescent sequencer (PE Applied Biosystems).

Maximum parsimony ("branch-and-bound" search) was used to reconstruct the evolutionary relationships between bacterial strains from near full-length 16S rRNA gene sequences (30). Sequences were manually aligned against a collection of 16S rRNA sequences based on conserved regions of sequence and secondary structure (19). Regions of ambiguous alignment were omitted from the subsequent analysis (1344 informative positions were retained). The reliability of the "best" phylogenetic tree was tested by performing bootstrap analysis (1000 replicates) with maximum parsimony (30), using a "branch-and-bound" search. In order to calculate phylogenetically independent contrasts (see below), a branch length of one unit was introduced between node H and the

terminal branches separating the identical 16S rRNA sequences of strains KBS-LC13 and KBS-LC14.

**Number Cell Yield & Growth Rate Determination**

Initial attempts to culture bacteria in a liquid basal salts medium, with succinate as a sole source of carbon and energy, failed due to an inability to reliably sub-culture strains. A complex medium (R2 broth, or R2B) supporting growth of all strains at a range of concentrations was used for numerical yield studies (26). Each liter of R2B contains: 0.5 g yeast extract, 0.5 g proteose peptone #3 (Difco, Detroit), 0.5 g casamino acids, 0.5 g glucose, 0.5 g soluble starch, 0.3 g sodium pyruvate (filter sterilized and added after autoclaving), 0.3 g $K_2HPO_4$, and 0.05 g $MgSO_4 \cdot 7H_2O$ (pH 7). Full strength (prepared as formulated above) medium was diluted 10-, 100-, 1000-, and 5000-fold to achieve a range of nutrient concentrations for numerical cell yield determination. All experimentation was initiated from freezer stocks to minimize spurious correlations resulting from differential adaptation of strains to laboratory culture conditions. Yield experiments were initiated by inoculating 10 mL of "full strength" R2B with a loop full of bacterial freezer stock. All cultures were grown at 25°C in 50 mL Erlenmeyer flasks placed on a rotary platform shaker (200 rpm). Starter cultures were allowed to reach stationary phase (two days for fast growers, three days for slower growers) and were then diluted 100-fold into 10 mL of fresh medium (conditioning cultures) at the concentration used for yield determination . Upon reaching stationary phase in conditioning cultures, bacteria were again diluted 100-fold into fresh medium (experimental cultures) at the concentration used for

yield determination. Samples (100 µL) were removed from experimental cultures at regular intervals, serially diluted in buffer (5 mM potassium phosphate, pH 7), and spread in triplicate on full strength R2 agar plates (R2A, Difco, Inc.). Viable cell counts were obtained from colony forming units (CFUs) enumerated on R2A plates, assuming that 100% of viable cells were able to form colonies. Numerical yields were calculated as the difference between the initial number of CFUs and the maximum number of CFUs per mL of medium. Growth rates were determined for each strain using linear least-squares regression of a minimum of four points defining the exponential phase of bacterial growth on a plot of CFUs ($\log_{10}$) versus time.

**Data Analysis**

The relationship between numerical cell yield and medium concentration was analyzed by simple linear regression and ANOVA. A full set of orthogonal contrasts was calculated to determine the nature of the relationship between numerical cell yield and medium concentration. The effects of medium concentration on numerical cell yields were also compared between strains for each medium concentration using principle component analysis (PCA). PCA was implemented using the 'PRINCOMP' routine in SAS/STAT version 8.1 (SAS Institute, Cary, NC) on the Windows 2000 (Microsoft, Redmond, WA) computer operating system. ANOVA was used to determine if significant differences existed between strains or rRNA operon copy number groups. Zero values were included in statistical calculations unless otherwise specified.

During comparative biological studies of organisms, biological species are often treated as independent sample observations to meet the assumptions of parametric statistical tests of significance. Phylogenetic relationships between evolutionarily related species violate assumptions of statistical independence and can result in spurious correlations (6). The method of phylogenetically independent contrasts transforms (or scales) comparative data based on the evolutionary relationships between pairs of species to meet the statistical assumptions of independence, normality, and homoscedasticity. We applied the method of Felsenstein (6) to correct for non-independence of data using organismal phylogenies inferred from 16S rRNA gene sequences. In the method of Felsenstein (6), pairs of independent contrasts are calculated between each species and its ancestral node with the assumption that any evolutionary change occurring past the node is *independent* of evolutionary change in other species pairs (21). Independent phylogenetic contrasts (6) were calculated with the COMPARE version 4.3 software package (20) and PCA was performed as described above.

## RESULTS

### Description of Bacterial Strains

Bacteria were selected for numerical yield studies based on colony appearance time and phylogeny. A wide phylogenetic distribution of bacteria was selected to ensure that a correlation between numerical cell yield and colony appearance time was an adaptation to selective pressures, rather than an ancestral artifact. The 12 bacterial strains selected for numerical yield studies represent a wide phylogenetic diversity, including the *Actinobacteria* and the α-, β-, and γ- classes of the *Proteobacteria* (Figure 5.1). Late (≥198 hr) appearing bacteria (identified by the strain designation 'LC' for late colonies) were constrained to the α-proteobacteria and *Actinobacteria*, while early (≤62 hr) appearing bacteria (identified by the strain designation 'EC' for early colonies) represented all major groups identified. Two of the late appearing isolates that appeared phylogenetically distinct with a single sequencing read (~350 nt), were identical *Azospirillum* spp. based on full 16S rRNA gene sequence analysis. The phylogenetically independent contrast method requires a bifurcated tree; therefore, zero branch lengths were manually lengthened one unit from node H to the terminal branch length of these two strains (Figure 5.1). All bacterial strains formed distinct isolated colonies on R2A agar plates, enabling easy enumeration.

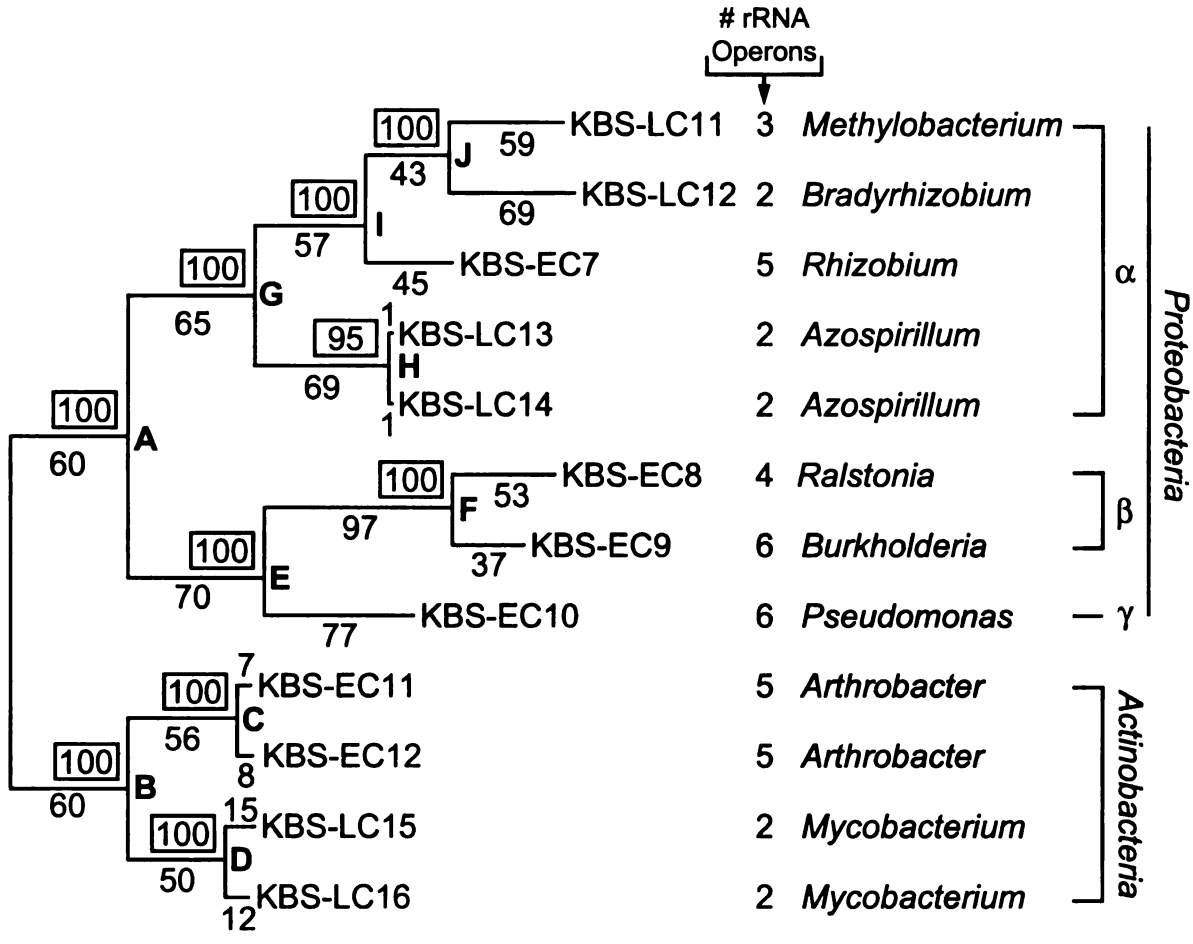


**FIGURE 5.1.** Phylogenetic relationships between the 12 bacterial strains used for numerical yield studies. Maximum parsimony tree based on comparison of 1344 nucleotide positions. Values adjacent to horizontal branches indicate the number of unique characters between nodes and termini; numbers in boxes above nodes indicate percentage of 1000 bootstrap replicates supporting each bifurcation; letters indicate ancestral nodes used for phylogenetically independent contrasts. Strain designations 'EC' indicate early appearing colonies (≤62 hr following plating); 'LC' indicates late appearing colonies (≥198 hr following plating).

The number rRNA operons per genome were experimentally determined for each bacteria strain used in numerical yield experimentation. Previous characterization of soil microbial communities revealed a correlation between colony appearance time (Chapter 3), rRNA synthesis capacity (Chapter 4), and rRNA operon copy number. If a trade-off exists between growth rate and numerical cell yield, rRNA operon copy number would provide a genotypic indicator of microbial fitness in the soil environment. Two populations of bacteria (6 strains each) were chosen based on colony appearance time that possessed many (4 – 6) and few (2 - 3) rRNA operons and represented a broad phylogenetic diversity (Figure 5.1). Bacteria with few rRNA operons were placed in phylogenetic groups including *Methylobacterium*, *Bradyrhizobium* and *Mycobacterium* that are recognized for slow maximal growth rates. Early appearing bacteria with many rRNA operons included strains in the *Burkholderia*, *Ralstonia*, *Pseudomonas, and Arthrobacter* genera that are recognized for rapid growth.

**Numerical Cell Yields & Growth Rates**

Batch cultures were used to determine effects of resource concentration on numerical cell yields of bacterial populations. The hypothesis tested was that late appearing bacteria with few rRNA operons would be more efficient at transforming resources into progeny. The theoretical model presented in Figure 5.2(A) predicts that early appearing bacteria with many (4-6) rRNA operons will have lower numerical cell yields than late appearing bacteria with few (2-3) rRNA operons due to decreased resource utilization efficiency over the broad range of

medium concentrations. The linear relationship (slope = 1) presented in this model indicates that the amount of resources required to produce a cell is constant, and therefore independent of medium concentration.

A one-to-one linear relationship between numerical cell yield and medium concentration was not evident for early or late appearing bacteria strains over a medium concentration range between 1:1 and 1:5000. While a significant linear relationship >1 exists between numerical cell yield and medium concentration for both early and late appearing bacteria strains, only 54% (early) and 58% (late) of increases in yield are explained by concomitant increases in medium concentration ($P < 0.0001$) between 1:1 and 1:5000 (Figure 5.2, B). The slope of the regression between 1:1 and 1:5000 medium concentrations is 1.81 for early appearing bacteria with many rRNA operons (open squares, dashed line) and 1.29 for bacteria with few rRNA operons (filled circles, solid line) (Figure 5.2, B). A test for homogeneity of slopes at the 99% confidence level indicates that the two linear slopes are not significantly different ($F_{1,6} = 1.68 \times 10^{-4}$), providing further evidence that numerical cell yields are not significant different over the range of medium concentrations tested.

The strength of the linear relationship between numerical yield ($R^2$) and medium concentration is negatively affected by non-growth values at the 1:5000 medium concentration. An apparent difference in numerical yield at 1:5000 between bacteria with many and few rRNA operons is deceiving since the average yield of strains with many rRNA operons reflects the growth of only three strains (Table 5.1).

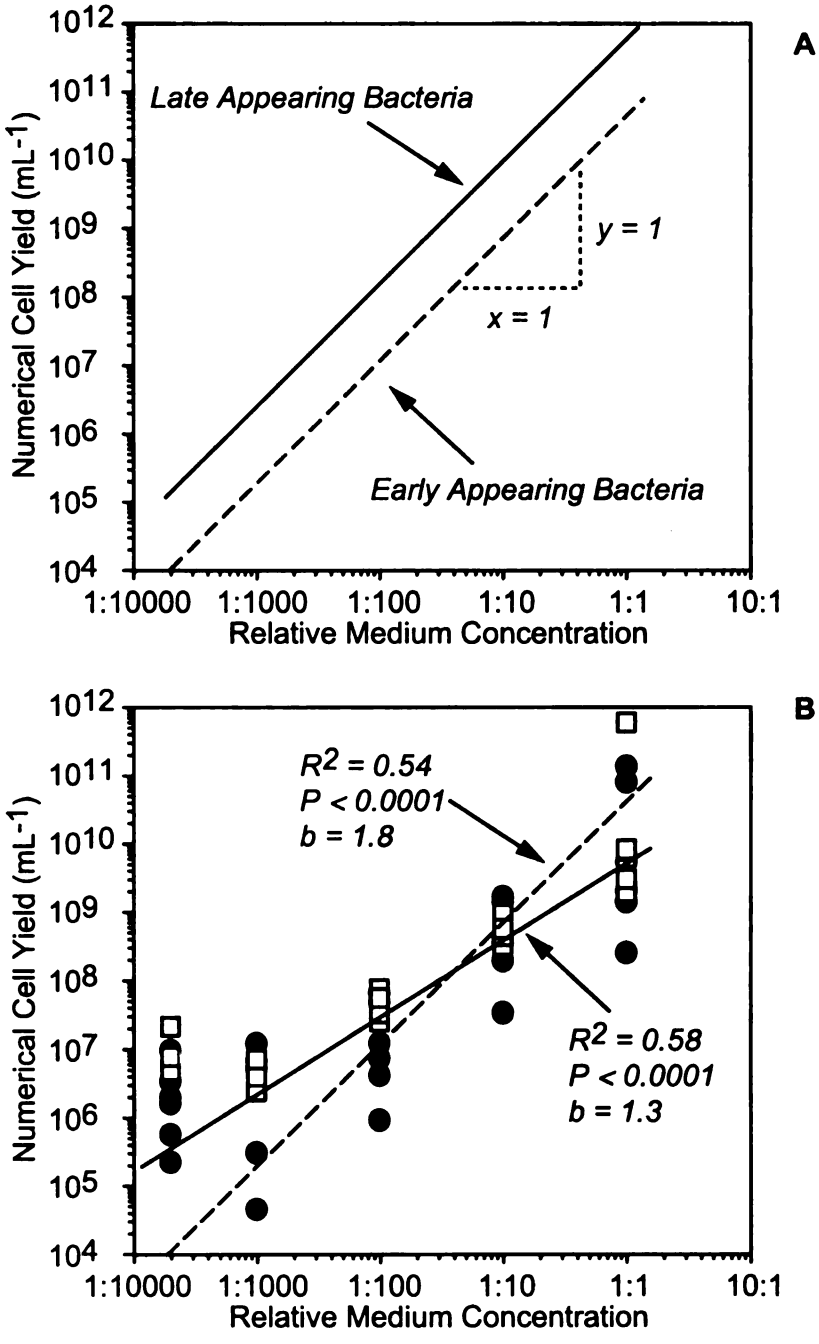


**FIGURE 5.2.** Relationship between numerical cell yields and medium concentration. (**A**) Theoretical relationship between numerical cell yield and medium concentration. A linear slope of one is presented for early (dashed line) and late (solid line) appearing bacteria. (**B**) Experimentally determined numerical cell yields. A linear regression was performed for bacterial strains grouped by early (open squares) and late (filled circles) colony appearance times over the range of medium concentrations. Yield is defined as the difference between the initial cell concentration and the maximum cell concentration. Error bars are the standard error of the mean. *P*-values indicate the probability that no relationship exists.

**TABLE 5.1.** Numerical cell yields[a] for the 12 bacterial strains used for experimentation.

| | | Medium Concentration[b] | | | | |
|---|---|---|---|---|---|---|
| | **Strain** | **1:1** | **1:10** | **1:100** | **1:1000** | **1:5000** |
| Multiple rRNA Operons | KBS-EC7 | $2.1x10^9$ | $6.1x10^8$ | $6.0x10^7$ | $4.3x10^6$ | - |
| | KBS-EC8 | $6.7x10^9$ | $3.5x10^8$ | $2.7x10^7$ | $6.0x10^6$ | $2.3x10^7$ |
| | KBS-EC9 | $8.5x10^9$ | $4.7x10^8$ | $4.1x10^7$ | $2.6x10^6$ | - |
| | KBS-EC10 | $8.9x10^9$ | $7.5x10^8$ | $3.5x10^7$ | $4.4x10^6$ | $5.2x10^6$ |
| | KBS-EC11 | $6.5x10^{11}$ | $9.8x10^8$ | $8.0x10^7$ | - | $8.2x10^6$ |
| | KBS-EC12 | $3.2x10^9$ | $6.1x10^8$ | $6.0x10^7$ | $7.6x10^6$ | - |
| Few rRNA Operons | KBS-LC11 | $2.7x10^8$ | $3.6x10^7$ | $4.4x10^6$ | $3.2x10^5$ | $2.4x10^5$ |
| | KBS-LC12 | $2.2x10^9$ | $1.8x10^9$ | $1.3x10^7$ | $1.3x10^7$ | $1.0x10^7$ |
| | KBS-LC13 | $1.5x10^{11}$ | $1.5x10^9$ | $5.2x10^7$ | $7.2x10^6$ | $1.7x10^6$ |
| | KBS-LC14 | $5.8x10^9$ | $3.8x10^8$ | $7.0x10^7$ | $5.6x10^6$ | $2.1x10^6$ |
| | KBS-LC15 | $8.6x10^{10}$ | $5.0x10^8$ | $9.7x10^5$ | - | $3.6x10^6$ |
| | KBS-LC16 | $1.5x10^9$ | $2.1x10^8$ | $7.8x10^6$ | $4.8x10^4$ | $6.0x10^5$ |

[a] Cells per mL culture medium. Numerical cell yields calculated as the difference between the initial and the maximum cell yield.

[b] Dilution of R2 medium used for yield determination

-, no growth

The proposed theoretical model describing the relationship between numerical cell yield and medium concentration of microbial growth predicted a one-to-one linear relationship between yield and substrate concentration (Figure 5.2, A). When the 1:5000 values are completely removed from regression analysis, a nearly one-to-one relationship exists between 1:1 and 1:1000 relative medium concentrations for individual bacterial strains (Figure 5.3). A nearly one-to-one relationship (1.06 ± 0.08) also exists for all strains (combined) when 1:5000 values are removed from regression analysis (data not shown). This linear relationship implies that the conversion of nutrients into progeny is constant between 1:1 and 1:1000 medium concentrations, supporting the proposed model.

Analysis of numerical yields for individual bacterial strains indicates some peculiarities at lower medium concentrations. For instance, the two strains that failed to grow at 1:1000 (KBS-EC11 & -LC15) produced positive yields at 1:5000 (Table 5.1). Numerical cell yields were also greater at 1:5000 than 1:1000 for three of the strains growing at both concentrations (KBS-EC8, -EC10, -LC16). It is notable that only 3 of 6 strains with many rRNA operons exhibited growth at the 1:5000 dilution of media, while all 6 strains with few rRNA operons demonstrated positive net growth (Table 5.1).

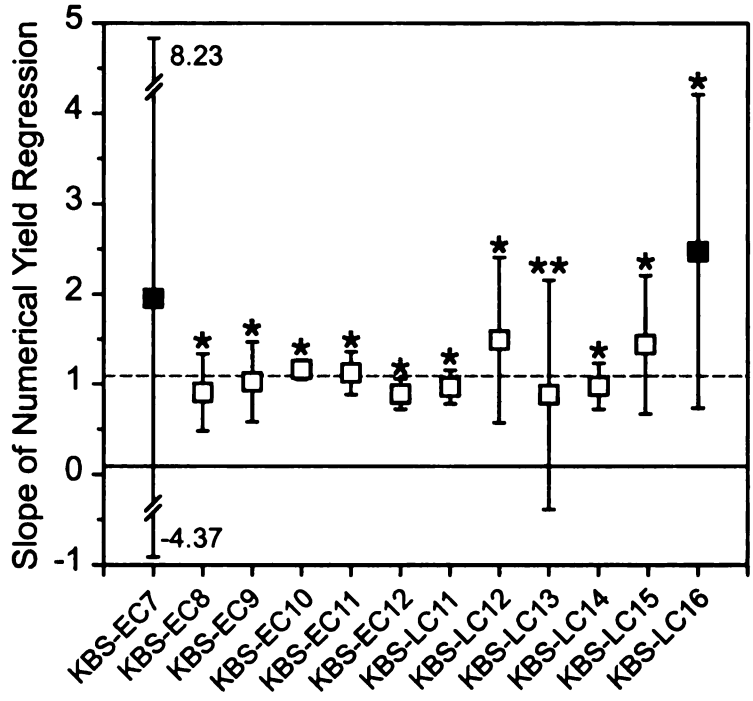


**FIGURE 5.3.** Change in numerical yields associated with increasing nutrient concentration between 1:1 and 1:1000. Values represent the slope describing the linear relationship between numerical cell yield and medium concentration on a $Log_{10}$-$Log_{10}$ scale. Error bars indicate the 95% confidence interval of the slope. Open squares indicate slopes obtained from regression between 1:1 - 1:1000 relative medium concentrations; filled squares, regression between 1:1 - 1:100; asterisks indicate the probability (*, $P < 0.05$; **, $P < 0.1$) of no relationship between numerical cell yield and medium concentration.

While differences in numerical yield were not clearly apparent, growth rates were easily distinguishable between populations grouped by colony appearance time (Figure 5.4). Early appearing bacterial strains with many (4 – 6) rRNA operons per genome grow at a mean population double time of 2.07 hr ($\mu$ = 0.34 $hr^{-1}$) while late appearing bacterial strains with few (2 – 3) rRNA operons grow significantly ($P < 0.01$) more slowly at a mean doubling time of 4.9 hr ($\mu$ = 0.15 $hr^{-1}$). Over the range of media concentrations where both populations of bacteria demonstrated growth, no trade-off is apparent between growth rate and numerical yield. Nevertheless, the growth rate of bacterial populations with many and few rRNA operons does indicate that the previous correlation between rRNA operon and colony appearance (Chapter 3) is also related to maximal growth rate.

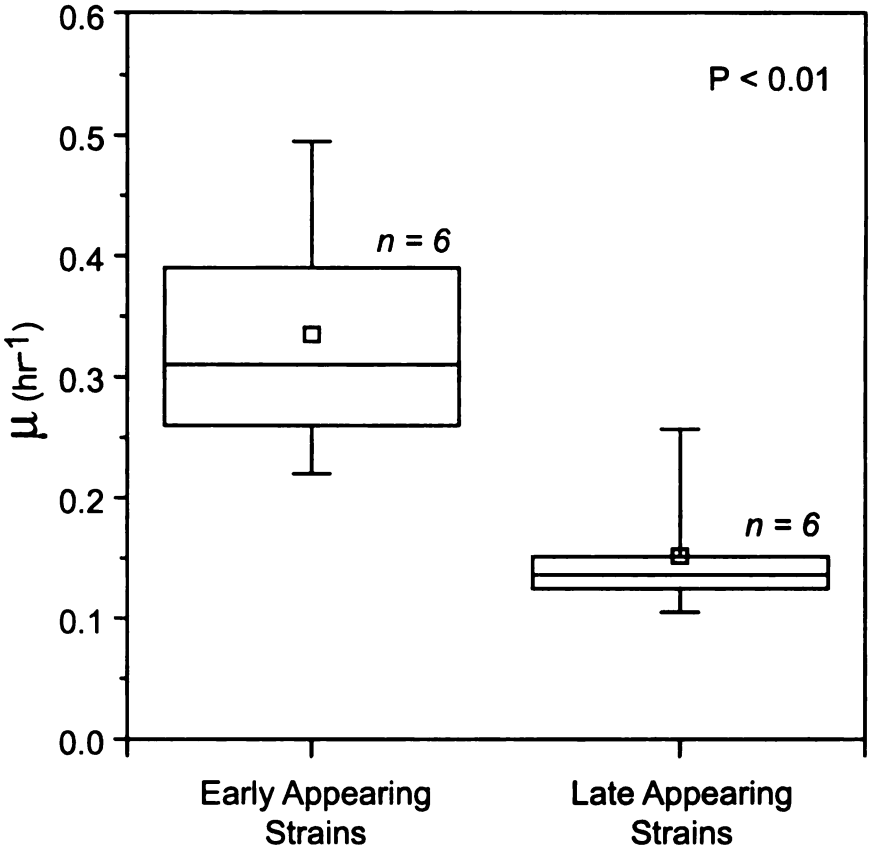


**FIGURE 5.4.** Growth rates of bacterial strains grouped by colony appearance time and rRNA operon copy number. Early appearing strains, 4 - 6 rRNA operons; late appearing strains, 2 - 3 rRNA operons per genome. Boxes contain the middle 50% of the sample distribution, squares indicate the sample mean, line dividing box indicates median value, and whiskers indicate the 95% confidence interval. Growth rates correspond to 2.4 hr (early) and 4.9 hr (late) mean doubling times.

**Phylogenetically Independent Contrasts**

The failure to demonstrate a trade-off in maximal growth rate and numerical yield led us to question whether grouping bacteria by rRNA operon copy number was masking differences between individual bacterial strains. Principal component analysis was used to visualize whether the apparent lack of differences in numerical yield could be attributable to factors other than colony appearance time. When grouped by medium concentration, the random distribution of points indicates that colony appearance time and individual phylogenetic groups explain little of the total variance in the data (Figure 5.5, A). The first two principle components each account for approximately 30% of the total variance, and loading factors of each principal component indicate no obvious effects due to a particular medium concentration. Both fast- and slow-growing groups of bacteria contain members of the α-proteobacteria and *Actinobacteria.* The α-proteobacteria and *Actinobacteria* do not cluster together, providing further evidence that the relationship between medium concentration and is not determined by a particular taxonomic group (Figure 5.5, A). Loading values indicate that the second principle component is affected by variability associated with numerical yields at the 1:1 medium concentration. Numerical yields for KBS-LC13 and -EC11 in full strength medium (Table 5.1) are above the 95% confidence interval and contribute to the increased variability associated with the second principle component, which also places these two stains as outliers (Figure 5.5, A).

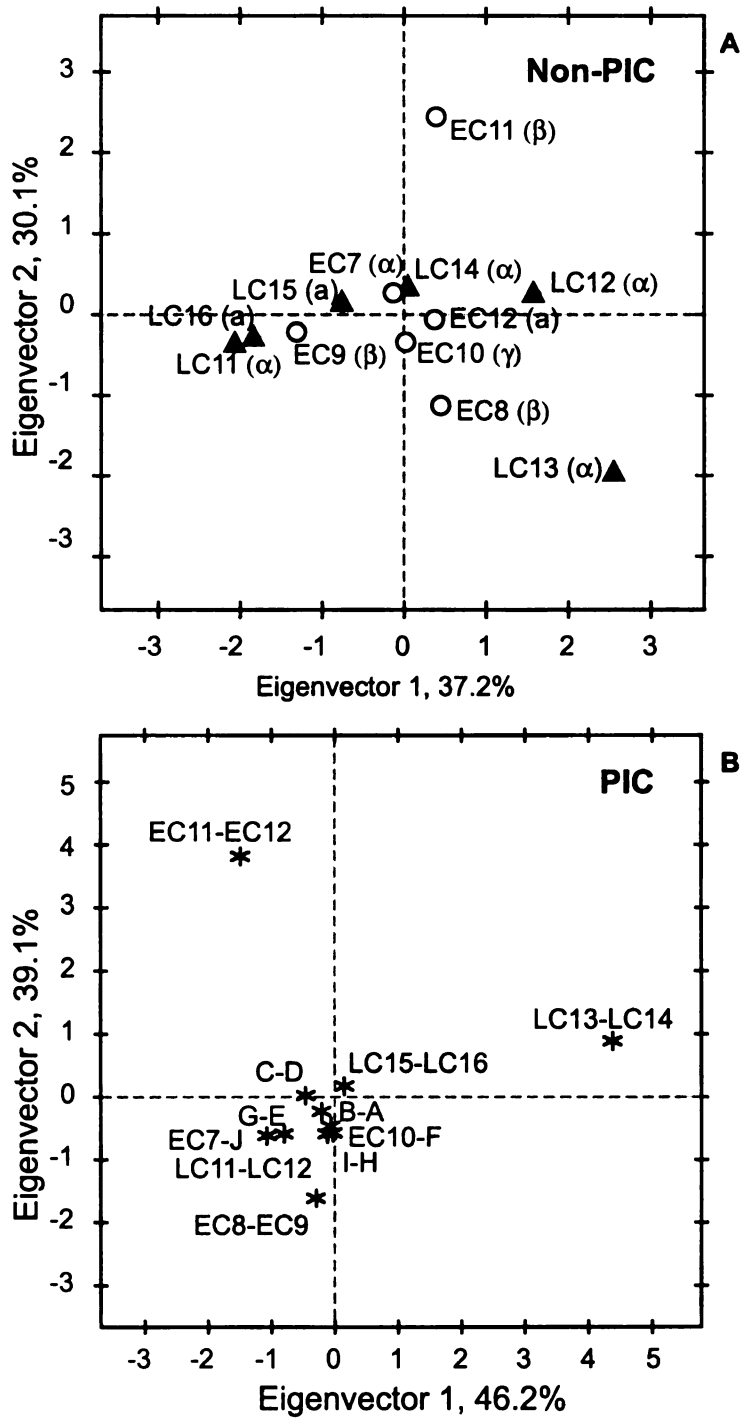


**FIGURE 5.5.** Principle component analysis of numerical cell yield for stains at each medium concentration. **(A)** variance associated with non-independent data for 12 bacterial strains analyzed. Strains with many rRNA (circles) and few rRNA operons (triangles) are indicated, including phylogenetic group affiliation (α-, β-, γ-proteobacteria; a, *Actinobacteria*). **(B)** scaled contrasts using Felsenstein's (6) method of phylogenetically independent contrasts (PIC). Refer to Figure 5.1 for bacterial strain and node information used to calculate PICs.

Principal component analysis was applied to data scaled by the method of phylogenetically independent contrasts (PIC) to determine the effects of phylogeny on the relationship between medium concentration and numerical yield (Figure 5.5, B). As observed for non-scaled data (Figure 5.5, A), PIC-scaled data are equally explained by the first two principle components (~40% each). Loading factors for variability associated with medium concentrations are equally distributed between the first two principle components. The effects of PIC-scaling are apparent with two outlying data points (EC11-EC12 & LC13-LC14) that increase the total variability above non-scaled data (Figure 5.5, B). The probability of detecting an adaptively significant trait is greater when two closely related strains differ in that trait. Therefore, a greater proportion of the total variability is attributable to the contrasts between EC11-EC12 (both *Arthrobacter* spp.) and LC13-LC14 (both *Azospirillum* spp.). The contrasts EC11-EC12 and LC13-LC14 each include one strain (LC13, EC11) that achieved a numerical yield above the 95% confidence interval for strains in full strength medium. It is difficult to ascertain whether these high yield values are actual physiological differences between closely related strains or experimental artifacts. If physiology follows organismal phylogeny inferred from 16S rRNA, it is probable that these high numerical yields are experimental artifacts and not true phenotypic differences. Nevertheless, high numerical yields in full strength medium for LC13 and EC11 do not affect the conclusion that a trade-off does not exist between numerical cell yield and growth rate.

## DISCUSSION

Under the range of conditions tested, a trade-off does not exist between numerical cell yield and maximal growth rate. Strains selected for this study were grouped based on colony appearance time on solid media, which has previously been correlated with rRNA operon copy number (Chapter 3). These results indicate that the correlation between rRNA operon copy number and colony appearance time on solid media is also related to maximal growth rate. Bacteria with many (4-6) rRNA operons demonstrated significantly faster growth rates ($\mu$ = 0.34 $hr^{-1}$) than bacteria with few (2-3) rRNA operons per genome ($\mu$ = 0.15 $hr^{-1}$) (Figure 5.4). These results indicate that in the absence of direct resource competition, slow-growing bacteria with few rRNA operons are capable of achieving numerical yields at levels equivalent to faster growing bacteria. The high abundance of slow-growing bacteria with few rRNA operons in the soil environment is unlikely related a greater resource utilization efficiency than late appearing bacteria with many rRNA operons.

The one-to-one linear relationship between numerical yield and medium concentration between 1:1 and 1:1000 is congruent with predictions based on Monod growth kinetics. However, it is clear that this relationship breaks down at lower concentrations of medium (Figure 5.2, B). One concern with numerical yield determination when cells are grown in low concentrations of liquid medium is the effect of substrate-induced lysis during plating on nutritionally rich media (24). It is possible that slow-growing cells were more susceptible to lysis during plating when grown at 1:1000 and 1:5000 medium dilutions due the a greater

osmotic change when plated on agar medium containing 1:1 concentration of R2B. However, bacterial populations failing to grow at the lowest medium dilutions were strains with high maximal growth rates. Spurious conclusions due to differential plating efficiencies among strains were therefore unlikely. Flow cytometry was attempted as a direct measure of numerical cell yield, however cell culture densities were below the level of detection (Coulter Channelyzer, electronic impedance detector).

The observation that only 3 of 6 fast growing strains were able to grow at a 1:5000 medium dilution indicates that a trade-off may exist between growth rate and the lowest medium concentration supporting growth. The half-saturation constant for bacterial growth ($K_S$) is often used to provide an estimate of bacterial substrate affinity. A 1:1000 dilution of the stock medium provides 2.7 μM glucose and 3.15 μM pyruvate, and a 1:5000 medium dilution provides only 0.54 μM glucose and 0.63 μM pyruvate. While these substrate concentrations are seemingly low relative to typical laboratory media, they are in the μM range that includes $K_S$ values reported for some bacteria growing on glucose. Comparatively, *Cytophaga johnsonae* C-21 grown in glucose-limited chemostats possesses $K_S$ values between 8.6 and 42.1 μM (11), while values for *E. coli* growing on glucose range between 22 and 500 μM (15). If these values provide an indication of $K_S$ for the collection of 12 strains examined, differences between fast- and slow-growing bacteria may be apparent at much lower concentrations of nutrients. Additionally, R2B medium contains complex substrates such as yeast extract and proteose peptone that can serve as carbon and energy

sources. Future experimentation should explore whether the inability of strains to grow at the 1:1000 1:5000 medium concentration results from carbon limitation or the absence of a particular trace element or vitamin.

Button (1991) proposed that oligotrophic bacteria are defined by the relationship between enzymatic substrate affinity and the intracellular catabolic capacity (5). Oligotrophic bacteria possess a high substrate affinity but low catabolic capacity, while copiotrophic bacteria possess low substrate affinity and high catabolic capacity. In this model, a trade-off exists between catabolic capacity and the ability to persist in environments where resources are perpetually scarce. When resources are abundant, high catabolic capacity is advantageous because bacteria can rapidly convert resources into new cells. However, during starvation high catabolic capacity is disadvantageous due to metabolic costs involved with transcriptional regulation of genes involved in substrate utilization. Both oligotrophic and copiotrophic bacteria can possess equivalent substrate affinity in this model, but the costs of maintaining high catabolic capacity make oligotrophic bacteria more fit in nutrient poor environments. Similar maintenance costs may be associated with the regulation of rRNA operons. Lower maintenance costs associated with few rRNA operons may indicate that bacteria with few rRNA operons are more efficient in nutrient poor environments. The observation that bacteria with few rRNA operons dominate soil environments (Chapter 4) and are capable of growing at low nutrient concentrations (see above) provides evidence that a low metabolic capacity is advantageous under conditions of perpetual resource scarcity.

Principle component analysis was used to explore the relationship numerical cell yields and medium concentration for each bacterial strain. One concern was that grouping bacterial strains by colony appearance time masked effects due to phylogeny. The failure of related bacterial groups or genera to cluster in PCA analysis indicates that numerical yields are not explained by organismal phylogeny (Figure 5.5). Variability associated with bacteria grouped by colony appearance time was equivalently distributed among all five medium concentrations evaluated. Spuriously high numerical yields at the 1:1 medium concentration for strains KBS-EC11 and –LC13 explain the high variability associated with these strains. Numerical yields should ideally be determined in at least triplicate cultures to determine the variability at the strain level. Practically, this was not readily feasible as each culture required 12 to 15 spread plates at each time point, for each medium concentration (experiments typically lasted 2 to 3 weeks, with 10 to 20 time points). Replication was achieved at the level of strains grouped by rRNA operon copy number, with six bacterial strains per group (2 groups, 6 strains each).

One weakness of the traditional comparative approach is the assumption of independence between sample observations. The samples under comparison are biological species related by evolutionary ancestry, and are not independent samples (6). A high probability exists that two closely related species possess similar adaptations to environmental selective pressures. Using the method of phylogenetically independent contrasts, we attempted to determine the effects of phylogeny between the numerical yields of bacterial strains used during

experimentation. Although PCA analysis of scaled numerical yield data is qualitatively similar to non-scaled data, phylogenetic effects are apparent for two contrasts in which one strain each exhibited an uncharacteristically high yield on full strength media (Figure 5.5, B). These results illustrate the potential effects phylogeny can have on conclusions of adaptive significance. If a closely related group of organisms is chosen for study, significant differences in phenotypic traits will be magnified when scaled in accordance with phylogeny. At the same time, there is a risk that no significant differences will be apparent since the species are phylogenetically related and likely share evolutionary adaptations (38). The highest probability of detecting a biological trait of adaptive significance is therefore achieved by studying a phylogenetically diverse collection of organisms, as were selected in the experiments described above.

A second concern with comparative ecological analysis of bacterial species is that fitness traits measured in the laboratory may reflect fortuitous pre-adaptations to laboratory conditions (27). For instance, a bacterial strain may be a superior competitor for a limited nutrient in the soil where moisture content rarely exceeds 12%, however it is an inferior competitor to other strains in liquid medium in the laboratory. One solution to this phenomenon is to evolve bacterial species under selective regimes in the laboratory, whereby the measurement of fitness traits will occur in conditions identical to the recent evolutionary history of the organism (37). Using this approach, Velicer & Lenski (1999) failed to demonstrate a trade-off in fitness between resource abundance and scarcity, but concluded that trade-offs may only exist once bacteria reach an adaptive peak in

their selective environment. Our inability to demonstrate a trade-off in maximal growth rate and numerical cell yield may indicate that the differences in growth rates are laboratory effects and not a reflection of different growth rates in soil. The correlation between rRNA operon copy number and growth rates on both solid and liquid media (of a variety of compositions) suggests that laboratory growth rates reflect differential growth rates in the soil (although most certainly at a different magnitude).

It is possible that neither numerical yield nor maximal growth receives strong selective pressures in the soil environment. The inability to demonstrate a trade-off in these traits in the laboratory demonstrates that competitive fitness in the soil is primarily determined by other physiological traits. If it is accepted that cellular division in the soil environment is rare (3, 10), then perhaps selective pressures are weighted for other traits such as starvation survival and nutrient affinity. Modeling of resource competition in phytoplankton communities indicates that nutrient affinity has a positive effect on relative fitness in non-equilibrium habitats receiving infrequent pulses of nutrients (8). Theoretical models simulating habitats receiving periodic fluxes of limiting nutrients also indicate high selective pressure for rapid recovery from starvation (14).

Bacteria with high affinity uptake systems and low metabolic costs may be better suited to the carbon-limited soil environment. Constitutive expression of rRNA during slow growth from experimentally introduced rRNA operons exerts a metabolic burden not realized during rapid growth in *E. coli* (28). Slow growth rates and the ability to grow at low nutrient concentrations indicate that bacteria

with few rRNA operons may be more efficient than bacteria with many rRNA operons during slow growth. Metabolic costs associated with many rRNA operons may be related to both constitutive expression of rRNA at slow grow rates and the maintenance of greater rRNA synthesis capacity. A recent survey of full genome sequences indicates that the number of regulatory genes per genome increases with genomic complexity (29). The synthesis of regulatory proteins imposes metabolic costs even if the genes are not expressed. Differences in numerical yield will likely be apparent between bacteria when the costs associated with increased rRNA synthesis capacity are realized by bacteria with many rRNA operons.

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# CHAPTER 6

## SUMMARY & CONCLUSIONS

Research described in this dissertation implemented a comparative approach to investigate the ecological implications of rRNA operon redundancy in heterotrophic bacterial populations from soil. The presence of many rRNA genes among a background of single copy genes, the central role of the ribosome in cellular metabolism, and the correlation between rRNA operon copy number and bacteria sharing similar ecological niches suggested that the number of rRNA operons per genome is an adaptively significant trait among the *Bacteria* (Chapter 1). Based on these observations it was postulated that rRNA operon copy number reflects the general ecological strategies of heterotrophic soil bacteria for responding to nutrient availability.

Mapping of rRNA operon copy number onto a bacterial phylogenetic tree indicates a strong correlation between organismal ancestry and rRNA operon copy number (Chapter 2). The correlation between rRNA operon copy number and ancestry is dependent upon the phylogenetic level under comparison. Bacteria with the same number of rRNA operons are found in divergent phylogenetic lineages, yet clear patterns are apparent at the strain, species, genus, and higher taxonomic levels. Closely related bacteria have a high probability of possessing the same number of rRNA operons per genome (Chapter 1, Figure 1.2). Commonly recognized groups such as the $\alpha$-class of the *Proteobacteria, Spirochaetes*, and *Planctomycetes* contain species that typically

possess ≤3 rRNA operons per genome; groups such as the β- and γ- classes of the *Proteobacteria* and *Clostridia* typically possess ≥4 rRNA operons per genome (Chapter 4, Table 4.1). Bacterial genera within these groups contain organisms with related numbers of rRNA operons per genome. For instance, the *Mycobacteria* genus within the *Actinobacteria* phylum contains species that possess ≤2 rRNA operons per genome. A neighboring group in the *Actinobacteria*, the *Streptomyces*, typically possess ≥4 rRNA operons per genome. Therefore, rRNA operon copy number can be inferred from phylogeny at many different phylogenetic levels, however, phylogeny cannot be inferred from rRNA operon copy number.

Soil microbial communities were used as model communities to investigate the effects of rRNA operon copy number on phylogenetically diverse bacterial populations. Bacterial populations from soil were separated based on colony appearance time on solid agar medium to determine whether rRNA operon copy number is correlated with bacterial physiology (Chapter 3). The number of rRNA operons per genome correlated with the time required for soil bacteria to form visible colonies on solid media. Bacteria appearing 2 to 3 days following plating possessed, on average, a significantly greater number of rRNA operons than bacteria appearing 10 to 12 days following plating (Chapter 3, Figure 3.1). Colony appearance time was considered a measure of both growth rate and lag time, which are functions of the rRNA synthesis capacity of a bacterium. Soil bacteria forming colonies early and late following plating represented a broad phylogenetic diversity, including members of six major

bacterial lineages: α-, β-, γ-classes of the *Proteobacteria*, *Cytophaga/Flexibacter*, *Actinobacteria* (high mol%G+C genomes), and *Bacillus* (low mol%G+C genomes) (Chapter 3, Figure 3.2). The relationship between rRNA operon copy number and colony appearance times demonstrates phenotypic effects associated with the number of rRNA genes in a genome.

The correlation between rRNA operon copy number, phylogeny (Chapter 2), and colony appearance time (Chapter 3) permitted investigation of microbial community dynamics in soil using nucleic acid based methodology. Phylogenetic probes targeting 16S rRNA were used to monitor changes in rRNA abundance of soil bacterial groups in response to amendment with succinate or water in soil microcosms (Chapter 4). Phylogenetic groups of bacteria with many rRNA operons (≥4) responded to succinate amendment in soil microcosms by significantly increasing in rRNA abundance and population size. The largest increases in rRNA abundance were observed in the *Actinobacteria* and β-proteobacteria immediately following amendment, accompanied by an increase in the number of early colony-forming populations (Chapter 4, Figure 4.2). Increases in both rRNA abundance and early colony-forming bacteria following succinate-amendment demonstrate that bacteria with many rRNA operons are capable of rapidly increasing cellular rRNA synthesis and initiating cellular division in response to nutrient amendment.

The most dominant microbial group in soil microcosms, the α-proteobacteria, did not increase in rRNA abundance following succinate or water amendment. The dominance of the soil microbial community by α-proteobacteria

with few rRNA operons suggests that a high capacity for rRNA synthesis may confer little advantage when resources are in low abundance. When resources are scarce, as in the soil environment, energetic costs associated with the maintenance of increased rRNA synthesis capacity may confer a fitness cost to bacteria with many rRNA operons.

An ecological trade-off in maximal growth rate and numerical cell yield was hypothesized as a mechanism explaining the abundance of slow-responding bacteria in soil (Chapter 5). A collection of soil bacteria, isolated based on colony appearance time and rRNA operon copy number, were grown over a 5000-fold medium concentration range to test the proposed hypothesis. No trade-off was observed between numerical yield and growth rate under the conditions tested. However, growth rates of bacteria with few (≤3) rRNA operons were significantly lower than bacteria with many (≥4) rRNA operons per genome. While an ecological trade-off was not readily apparent, the failure of 50% of early appearing strains to grow at the lowest medium concentration may indicate that bacteria with few rRNA operons are more efficient at converting limiting resources into progeny at low nutrient concentrations. The inability of bacteria with many rRNA operons to grow at the lowest medium concentration suggests that maintenance of increased rRNA synthesis capacity imposes a metabolic burden when resources are perpetually scarce. An alternative hypothesis is that differences in the relative fitness of bacteria with many and few rRNA operons at low resource concentrations resulted from trace element or vitamin limitations and not carbon limitation alone. Future experimentation should explore the

relationship between differential nutrient limitations at low medium concentrations. In addition, medium concentrations lower than 1:5000 may preclude the growth of a greater proportion of bacteria with many rRNA operons, providing further evidence for potential metabolic costs associated with high rRNA synthesis capacity.

Together these results indicate that rRNA operon copy number reflects the rRNA synthesis capacity of a bacterium. Bacteria with many rRNA operons are capable of rapidly increasing rRNA synthesis and growth following increases in nutrient availability. An increased capacity for rRNA synthesis does not come at the cost of decreased resource utilization efficiency. Bacteria with both many and few rRNA operons possessed equivalent numerical yields over a wide range of nutrient concentrations despite significant differences in growth rates. Slowly growing bacteria with few rRNA operons comprised the largest fraction of the soil microbial community, indicating that a low capacity for rRNA synthesis is correlated with competitive success in soil. The low abundance of bacteria with many rRNA operons in soil may result from metabolic costs associated with the maintenance of a higher capacity for rRNA synthesis.

Trade-offs in bacterial competitive abilities are traditionally associated with performance under resource abundance versus scarcity. Attempts to demonstrate a trade-off in competitive ability under these resource conditions have historically focused on differences in growth rate parameters modeled by Monod growth kinetics. The failure to demonstrate a trade-off in $\mu_{max}$ and $K_s$ results from the inseparable relationship between these two parameters. $K_s$

does not represent enzymatic nutrient affinity, but rather the overall metabolic capacity of a bacterium to transport and utilize nutrients for cellular material and energy. High metabolic capacity confers rapid growth rates and short lag times. rRNA operon copy number is a genetic indicator of central metabolic capacity that reflects the ability of bacteria to rapidly increase cellular metabolism and growth when nutrients are abundant. Bacteria with either many or few rRNA operons are capable of utilizing resources in low abundance, however bacteria with few rRNA operons are metabolically limited in the rate in which resources are utilized. A trade-off in competitive ability between bacteria with many and few rRNA operons will only be realized under conditions of low resource availability when the burden of maintaining increased metabolic capacity becomes apparent among bacteria possessing many rRNA operons. These observations support the hypothesis that rRNA operon copy number is an adaptively significant trait indicating the ecological strategies of heterotrophic bacteria in soil.

# APPENDIX A

## BASAL SALTS MEDIUM RECIPE

| Minimal Salts | Final Concentration (mM) |
|---|---|
| $Na_2HPO_4$ | 5 |
| $KH_2PO_4$ | 5 |
| $(NH_4)_2SO_4$ | 5 |
| $MgSO_4$ | 1 |

Prepare each solution separately and autoclave.

| Trace Element Solution | Final Concentration (μM) |
|---|---|
| HCl (conc.) | 70 mM |
| $FeSO_4 \cdot 7H_20$ | 7.5 |
| ZnCl2 | 0.5 |
| $MnCl_2 \cdot 4H_20$ | 0.45 |
| $H_3BO_3$ | 0.1 |
| $CoCl \cdot 6H_20$ | 0.8 |
| $CuCl_2 \cdot 2H_20$ | 0.010 |
| $NiCl_2 \cdot 6H_20$ | 0.1 |
| $Na_2MoO_4 \cdot 2H_20$ | 0.2 |

Dissolve the ferrous sulfate completely in HCl before adding distilled water to the final volume. Add the metal salts in the given sequence and make sure that each one has dissolved completely the next one is added. Note that this is an acidified solution and can be autoclaved. Do not filter sterilize as metals will precipitate on the filter. Divide into working aliquots and autoclave, store away from light at 4°C.

| Vitamin Solution | Final Concentration (μg/L) |
|---|---|
| Pyridoxine HCl | 50 |
| Thiamine HCl | 25 |
| Nicotinic acid | 25 |
| p-Aminobenzoic acid | 25 |
| Biotin | 10 |
| Folic acid | 10 |
| Pyridoxal phosphate | 0.5 |
| Riboflavin | 25 |
| Thioctic acid | 25 |
| Pantothenic acid | 25 |

Add in the given sequence and make sure that each one has dissolved completely before the next one is added. Filter sterilize with a 0.22 $\mu m^3$ cutoff filter and dispense into working volumes. Do not autoclave. Store away from light at 4°C.

# APPENDIX B

## RIBOSOMAL RNA COPY NUMBERS OF SOIL BACTERIA COLLECTION

| | Early (<62 hr) Appearing Bacteria | | Late (>198 hr) Appearing Bacteria | |
|---|---|---|---|---|
| α-*Proteobacteria* | **Strain** | **n** | **Strain** | **n** |
| | T1 2.14 | 5 | T1GX 3.15 | 1 |
| | T1B 2.9 | 4 | T1GX 3.9 | 2 |
| | T1R 2.9 (KBS-EC7) | 5 | T1GX 4.2 | 2 |
| | | | T1GX 4.4 | 2 |
| | | | T1PX 3.13 (KBS-LC11) | 3 |
| | | | T1PX 3.14 (KBS-LC13) | 2 |
| | | | T1PX 3.16 | 4 |
| | | | T1PX 3.18 | 3 |
| | | | T1PX 4.1 (KBS-LC14) | 3 |
| | | | T1PX 4.2 | 2 |
| | | | T1YX 4.1 | 3 |
| | | | T1YX 4.2 | 2 |
| | | | T1YX 4.4 | 2 |
| | | | T1YX 4.9 | 2 |
| β-*Proteobacteria* | **Strain** | **n** | | |
| | T1 2.2 (KBS-EC8) | 4 | | |
| | T1 2.8 | 5 | | |
| | T1 2.11 (KBS-EC9) | 6 | | |
| | T1 3.2 | 5 | | |
| | T1B 2.13 | 4 | | |
| | T1B 2.15 | 5 | | |
| γ-*Proteobacteria* | **Strain** | **n** | | |
| | T1 2.1 | 6 | | |
| | T1 2.4 | 6 | | |
| | T1 2.5 | 4 | | |
| | T1 2.6 | 4 | | |
| | T1 2.7 | 7 | | |
| | T1 2.9 | 6 | | |
| | T1 2.10 | 5 | | |
| | T1 2.15 | 6 | | |
| | T1 2.17 | 6 | | |
| | T1 2.19 | 6 | | |
| | T1 3.1 (KBS-EC10) | 6 | | |
| *Actinobacteria* | **Strain** | **n** | **Strain** | **n** |
| | T1B 2.6 | 5 | T1GX 3.14 | 2 |
| | T1B 2.12 (KBS-EC11) | 5 | T1PX 3.15 (KBS-LC15) | 2 |
| | T1B 2.14 | 5 | T1PX 5.1 (KBS-LC16) | 2 |
| | T1R 2.1 | 5 | T1YX 4.9 (KBS-LC12) | 2 |
| | T1R 2.8 | 5 | T1YX 4.10 | 1 |
| | T1R 3.1 (KBS-EC12) | 5 | | |