BACTERIAL GROWTH EFFICIENCY: ASSESSMENT IN TERRESTRIAL ECOSYSTEMS

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ABSTRACT

BACTERIAL GROWTH EFFICIENCY: ASSESSMENT IN TERRESTRIAL ECOSYSTEM AND INFLUENCING FACTORS

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Bacteria in soil are responsible for converting labile root exudates into microbial biomass that is more stable. This transformation is important for stabilizing soil organic carbon (SOC) and maintaining soil fertility. Bacterial partitioning of SOC into biosynthesis of new biomass or mineralization to carbon dioxide is defined as bacterial growth efficiency (BGE). BGE is also an integral component of models that simulate carbon dynamics in soil. However, variation in BGE in terrestrial ecosystems is not well understood, nor are environmental factors that influence it. In fact, BGE is often assumed to be constant. This dissertation explores BGE in four terrestrial biomes at the Kellogg Biological Station Long Term Ecological Research site and assesses factors that may influence BGE.

BGE is calculated from bacterial production (BP) and respiration (BR) using the formula BGE = BP/(BP+BR). In terrestrial ecosystems, these parameters are often measured using a single radiolabeled substrate, which masks any influence of BGE specific to resources available in the soil. We developed a method that permits BGE measurements to reflect the nutrient status of the soil. BP was measured as ³H-leucine incorporation rate into protein and BR as oxygen consumption rate, both without exogenously added substrates. Using this method, variation of BGE was assessed for soils collected from deciduous forest and three different croplands. We showed that BGE was not constant, but varied from 0.23 to 0.63. Bacterial communities in soils from soybean monoculture cropland tended to have a higher BGE than those in deciduous forests or rotational cropland soils. BGE in cropland soils exhibited a large seasonal variation not observed in forest soils.

BGE can also be influenced by the composition of bacterial communities, as different bacterial species have different energy and growth requirements. However, it is challenging to link a general function such as carbon transformation to the structure of the bacterial community because of high functional redundancy. Using 16S rRNA gene surveys of rotation cropland and forest soils, it is proposed that the efficiency of bacterial communities can be predicted. In addition to identification of the types of bacteria in the soil sample, 16S rRNA gene surveys also provide a glimpse of the lifestyle of the bacteria. The identity of the bacteria provides an estimate of the number of 16S rRNA genes in the genome, which can then indicate the ecological strategy of the bacteria. Based on the ecological strategy, the growth efficiency can be predicted. The rotation cropland and forest soils have different community composition but the overall 16S rRNA gene copy number is the same, consistent with the observation that these two communities have similar growth efficiencies.

Inclusion of measured values of BGE into DAYCENT, a model simulating nutrient cycling in soils, revealed the sensitivity of the model to changes in BGE. When simulating carbon dynamics in a mock ecosystem, decreasing the default BGE value of 0.45 to the average BGE in forest soils, 0.35, reduced the active carbon fraction by 22%. This led to a 5% reduction in the predicted total soil carbon at equilibrium. Therefore, site-specific BGE is important for improving the predictive capacity of SOC models, especially when investigating the effects of changes in climate, soil edaphic properties and land management practices on labile SOC transformations.

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CHAPTER 1

BACTERIAL-DRIVEN SOIL CARBON SEQUESTRATION

Bacterial Driven Carbon Transformations

Soil organic carbon (SOC) makes up the largest terrestrial reservoir of carbon with 1550 Pg carbon, almost three times more than the amount of carbon in vegetation (36). Some of the SOC is vulnerable to changes in climate, particularly moisture and temperature, and especially to land use change. The conversion of forest to intensively managed cropland can result in the loss of up to 50% of the stored carbon (72). Due to the large amount of carbon stored in soil, a small change in soil carbon stock can have a significant impact on the amount of carbon in the atmosphere, which is currently 760 Pg (36). Historical change in land use during the years 1850 to 2000 was estimated to release up to 156 Pg CO₂-C, second only to industrial sources of CO₂ (31). Not only does the loss of soil carbon increase atmospheric CO₂, it also decreases soil fertility (71).

Replenishing the carbon lost from soil following deforestation would offset some of the CO_2 released to the atmosphere (37). With the decreasing capability of the ocean to serve as a carbon sink, more attention is being focused on carbon sequestration in soil (30). Promoting carbon sequestration in soil is also important for improving soil fertility and preventing soil degradation. Soil degradation is one of the most challenging problems associated with terrestrial carbon loss because degraded soils lead to decreases in primary productivity and are difficult to rejuvenate (23). Cropland that was converted back into grassland takes decades to accumulate SOC and does not necessarily return to the pre-agriculture SOC content (47). The amount of

carbon that can be stored in soil is determined by the amount of carbon input, primarily from photosynthesis, and the amount of carbon lost during decomposition of SOC by microbes. Therefore to increase soil carbon there has to be an increase in primary productivity, a decrease in carbon loss during decomposition, or both.

Plant-derived carbon (litter material and root exudates) is the major input of organic carbon into soils. Some of this carbon is directly assimilated by microbes and incorporated into biomass (Figure 1-1, solid arrows). More recalcitrant plant polymers such as lignin are degraded with extracellular enzymes into labile, low-molecular weight substances that are subsequently consumed by microbes (Figure 1-1, dashed arrows). Simultaneously, some compounds undergo chemical interactions to form humic acids and become physically protected in soil aggregates (Figure 1-1, grey arrows). This carbon pool is known as passive carbon and it is not typically accessible for decomposition by bacteria. The structure of bacterial biomass and extracellular polysaccharides that allow strong interactions with soil particles make bacterial products a crucial component of SOC formation, especially in assisting with humic formation (41, 42). In fact, recalcitrant carbon in the passive carbon pool is dominated with carbon of microbial origin (32). The central role of bacterial activity in soil organic carbon formation is further supported by the observation that molecular structure of soil carbon is similar across all land management practices (21).

Carbon in the passive carbon pool can become accessible to active microbes. This often happens when cropland is tilled or there is an increase in soil moisture (22). Tillage homogenizes aggregates while moisture increases diffusion rate of low molecular weight substances, making the carbon accessible to bacteria (Figure 1-1, dotted arrow). Tillage also increases oxygen availability, which can increase decomposition rate (4). No-tillage practices

can lead to the recovery of more than 10% of soil carbon in the surface layer (45). Additionally, when carbon input increases but the bacteria are still limited for nutrients such as nitrogen, extracellular enzymes can be produced to mine for the limiting nutrient and enhance decomposition (6). Therefore, bacterial communities in soil are also responsible for the loss of passive soil carbon.



Figure 1-1: Bacteria play a central role in soil carbon transformations. Plant-derived carbon (litter material and root exudates) serves as substrates for bacterial growth. Solid black arrows represent decomposition that immobilizes carbon. Dashed black arrows represent degradation by extracellular enzymes. Solid grey arrows represent chemical and physical interactions between lignin and bacterial products with soil particles to form stable passive carbon. Dotted black arrow represents the decreased protection of passive carbon that releases labile carbon for decomposition by bacteria.

The efficiency with which bacteria transform carbon substrates into biomass can be measured as bacterial growth efficiency (BGE). BGE is defined as the fraction of the total carbon consumed that is incorporated into biomass (13). Aerobic heterotrophic bacteria assimilate plant-derived carbon both to synthesize biomass and to respire to CO_2 for energy (Figure 1-2). A more efficient bacterial community will be able to incorporate a greater percentage of carbon consumed into biomass material and so increase the abundance of material for passive carbon formation. Therefore, soils with more efficient bacterial communities have higher potential to

become a carbon sink and are more capable of maintaining soil fertility. More efficient carbon transformations by the bacterial communities will also preserve more energy in the ecosystem for higher trophic level organisms.



Figure 1-2: Soil organic carbon serves as both a carbon and energy source for aerobic heterotrophs in soil.

Bacterial Growth Efficiency

During aerobic growth, BGE is determined by two parameters of bacterial activity – the synthesis of biomass, also known as bacterial production (BP), and bacterial respiration (BR) (Figure 1-2). Hence, BGE is calculated as BP/(BP+BR) (13). In pure cultures of bacteria, BGE is found to be influenced by environmental conditions and intrinsic factors (61). At ranges of resource availability typically provided in laboratory media, BP and BR are strongly coupled. This means that BGE remains constant as substrate concentration varies (51). For example, BGE

for *Aerobacter cloacae* stays within the range of 0.56 to 0.6 when grown in a medium containing glucose as the sole carbon and energy source (26). However, at very low substrate concentrations, BP and BR become uncoupled, causing BGE to decrease with substrate availability (69). This uncoupling of BP and BR is due to the constant requirement for maintenance energy within a bacterium. Maintenance energy is the energy used to support non-growth processes such as cellular integrity, ion gradients and endogenous metabolism (52). Uncoupling of BP and BR can also occur at high carbon substrate concentrations, when growth becomes limited by nutrients such as nitrogen or phosphorus (43). Under these conditions, bacteria frequently respire the excess carbon in a futile cycle known as energy spilling (56).

In addition to resource availability, BGE is also dependent on the composition of the growth medium because this influences the energy required for biomass synthesis. For example, *Streptococcus bovis* is found to exhibit a 40% increase in growth efficiency when amino acids are provided in the growth medium (55). Amino acid biosynthesis requires between 12 and 74 molecules of ATP for each amino acid synthesized, therefore more of the substrate carbon is required for respiration (BR) leading to a decrease in BGE. BGE is also influenced by the amount of free energy available in the growth substrate. A low energy substrate such as methane will provide less carbon for growth after the energy demand is fulfilled (51).

Intrinsic factors are determined by the genetic makeup of an organism. There are two ways in which differences in genetic capability can influence BGE. One is the growth-related energy requirement and the other is the maintenance energy requirement. An organism that uses a catabolic pathway that yields more energy will be able to allocate more carbon for growth instead of ATP synthesis. *Streptococcus faecalis*, utilizing the Embden-Meyerhof pathway that yields 2 moles ATP/mole glucose, has a higher BGE than *Leuconostoc mesenteroides* using the

Entner-Doudoroff pathway that yields 1 mole ATP/mole glucose (5). It has been proposed that different organisms have different maintenance energy requirements, but more research is required to determine the interspecies variability (57, 73).

Growth-related energy requirements can also be decreased by reducing the cost of biomass synthesis. The most energetically expensive cellular process is translation, the process of protein synthesis. With four ATP molecules required for the formation of each peptide-bond, protein synthesis consumes up to 60% of cellular ATP during exponential growth (66). Different bacteria can have ribosomes with different translational performance. Fast-growing bacteria have higher translational power, which is the rate of functional protein synthesized per unit translational machinery (15). In *E. coli*, different isolates can have ribosomes with different efficiencies, as measured by the rate of amino acid incorporation per ribosome (54). As protein synthesis is such an energy demanding process, bacteria with ribosomes that can synthesize more functional proteins per unit energy are predicted to be more efficient, especially in environments with limited resources (15). This hypothesis is briefly explored in Appendix A using *E. coli* strains having ribosomes with different translational performances.

The sensitivity of growth efficiency to both environmental factors and genomic make-up can result in a large variation in BGE of bacterial communities in the environment. Additionally, the quality and concentration of resources often fluctuate in the environment, which can limit growth and cause uncoupling of BP and BR. It is therefore not surprising that where BGE has been intensively explored, such as in the aquatic environment, it is found to range from 0.05 to 0.6 (14). Aquatic BGE is mainly driven by changes in quality and quantity of dissolved organic substrates. Using chlorophyll as an indicator of system productivity, Lopez-Urrutia showed that BP is sensitive to resource availability but BR remains the same. Hence, the variation of BGE is

driven by the capability of bacterioplankton to incorporate substrate into biomass (44). Consequently, low BGE is a characteristic of oligotrophic lakes with low primary productivity (2, 10). The influence of substrate quality becomes more important in eutrophic environments where more labile carbon promotes increased efficiency (3). Due to the central role of bacterioplankton in carbon cycling in the aquatic system, high BR becomes a significant contribution to oceanic CO₂ flux into the atmosphere (11).

Growth efficiency in terrestrial ecosystems is not as well explored as in aquatic ecosystems because the heterogenous nature of soil makes measuring growth efficiency difficult. Additionally, terrestrial growth efficiency measurements are commonly based on a single substrate, typically applied at concentrations not commonly found in soil (Tables 1-1 to 1-3). Early measurements of microbial growth efficiency (MGE) using radiolabeled glucose found that MGE varies with the substrate concentration applied to the soil and the incubation time (9). This results in MGE measurements that are more reflective of the experimental perturbation than the nutrient status of the soil. Furthermore, MGE measurements using a single substrate represent the efficiency of microbes that can assimilate and mineralize the added substrate, rather than the entire active community. I adapted a method from aquatic ecology that does not require addition of exogenous substrate (Chapter 2). This method of measuring growth efficiency reflects influences from the soil environment and the overall activity of the bacterial community.

Several measurements of MGE have been reported for terrestrial biomes. However, the sensitivity of MGE to the substrate chosen by the investigators makes cross-site comparisons less meaningful (68). Using a substrate that can be utilized by most bacteria, such as ammonium nitrate, Schimel calculated MGEs for grasslands and croplands using three different C:N ratio. The grassland soils tended to have microbes that are more efficient (59). However, without more

growth efficiency measurements for a wider range of terrestrial biomes, factors that regulate growth efficiency in the terrestrial ecosystem cannot be determined. For example, the influence of land management practices on microbial-driven carbon transformations requires cross-site comparisons. Focusing on the dynamics of bacterial growth, we measured BGE in four terrestrial biomes at the Kellogg Biological Station Long Term Ecological Research (KBS LTER) site to identify factors that can regulate BGE in terrestrial ecosystems (Chapter 3).

Soil Bacterial Communities

Transformations of soil carbon are common functions for all heterotrophic microbes in soil. Unlike specialized functions such as denitrification or ammonia oxidation, there is no single functional gene that links CO_2 production by heterotrophs to the structure of microbial communities (24). In fact, while the diversity of methane-oxidizing bacteria is correlated with the level of methane oxidation (a specialized metabolic function), there is no correlation between heterotroph diversity and CO_2 production (a broadly distributed metabolic pathway) (40). An extensive study of bacterial community diversity in 98 soils across America found that pH was the only explanatory factor for differences in bacterial diversity (18).

Yet it is well documented that community compositions change in response to different substrate types and, in some cases, the change influences carbon mineralization rate (17, 67). This suggests that in order to study the influence of changes in community structure on carbon transformations, it is important to first identify bacterial groups that respond to different substrate availability and type (33). Changes in community structure indicate that when labile substrate becomes available carbon mineralization rate increases; this is often correlated with increased abundance of fast-responding bacteria like Gammaproteobacteria and Firmicutes (12).

Soil Texture	Substrate / Nutrients	Factors	Method	MGE	Ref.
Silt loam	Glucose	Low grazing		0.57 - 0.58	
(23% clay, 0.5% orgC)	(800 µg C/g soil)	High grazing	$Y_S/Y_B/Y_C$	0.24 - 0.65	(20)
Fine sandy loam	¹⁴ C-Glucose	Low grazing	(S)	0.51 - 0.59	(20)
(18% clay, 1.8% orgC)	(1g C/g soil)	High grazing		0.27 - 0.68	
Sandy loam (1.6% orgC) Silt loam (2.2% orgC)	¹⁴ C-Glucose, N, P (36 - 2304 μg C/g soil)	Glucose concentration	$Y_{R}(L)$	0.56 - 0.72	(8)
	¹⁴ C-Glucose (30-300 µg	Glucose concentration, Time	$Y_{B}(S)$	0.9	(9)
Silt loam (1.3% totC)	C/g soil) C:N:P = 150:10:1		$Y_{B}(L)$	0.57 - 0.75	
Black soil (49% clay, 2.1% orgC)	¹⁴ C-glucose (937 μg C/g soil), C:N = 25:1	None*	$Y_{R}(L)$	0.63	(64)
Clay loam	¹⁴ C-Glucose		$Y_{S}(S)$	0.91 - 0.92	(10)
26% clay, 1.3% orgC) (0.01 μ g C/g soil) Plants, T	Plants, Time	$Y_{S}(L)$	0.67 - 0.72	(48)	
Eutric Cambisols (3% totC)	¹⁴ C-Glucose (1 μM - 10 mM)	Glucose concentration	Y _R (S)	0.92 - 0.98	(28)
Silt loam (26% clay, 1.6% orgC)	¹³ C-Glucose (800 μg C/g soil)	F:B ratio, C:N ratio	$Y_{S}(S)$	0.59 - 0.62	(70)
Silt loam	Cellobiose (420 µg C/g soil)	Temperature, Time	$Y_{R}(L)$	0.60 - 0.80	(65)
Clay loam	¹⁴ C-Wheat straw	Straw application	$Y_{C}(L)$	0.05 - 0.43	(29)
Fine clay (51% clay, 2.1% totC)	Rice straw	C:N ratio, oxic status, temperature	Y _C (L)	0.30 - 0.61	(16)
Sandstone, loamy silt, fine shale	¹⁵ NH ₄ SO ₄ (1 µg N/g soil)	Soil texture	$Y_{B(N)}(L)$	0.25 - 0.59	(59)

Table 1-1: Microbial growth efficiencies (MGE) in agricultural soils.

Soil Texture	Substrate / Nutrients	Factors	Method	MGE	Ref.
Soddy-podzolic (1.9% orgC) Gray forest (2.3% orgC)	Glucose (4 mg C/g soil), N, P (C:N:P = 1:10:1 to 1:30:1) Sterilized soil	Substrate, Soil type	Y _R (S)	0.80 - 0.95	(7)
Silt loam (2.4% totC)	¹⁴ C-Glucose (0.0009 - 260 μg C/g soil), N, P	Glucose concentration	Y _B (S) Y _B (L)	0.5 - 0.85 0.4 - 0.61	(62)
Sandy loam (12% Clay) Clay (42% Clay)	¹⁴ C-Glucose (300 μg C/g soil), ¹⁵ N (20 μg N/g soil), ³² P	Soil texture, Moisture, Time	Y _S (S) Y _S (L) Y _S (S) Y _S (L)	0.78 - 0.86 0.61 - 0.67 0.80 - 0.88 0.64 - 0.71	(74)
Sandy silt (1% totC)	¹³ C Cellulose (495 mg C/g soil)	Nutrient (C:N:P = 80:8:1)	$Y_{B}(L)$	0.21 - 0.36	(19)
Course loam (2.89% orgC)	¹³ C-Acetate, ¹⁵ N-NH ₃ , vapor	Moisture, Method	$Y_{B}(L)$ $Y_{B(N)}(L)$	0.46 - 0.62 0.24 - 0.58	(27)
Sandstone, Loamy silt, Fine shale	¹⁵ NH ₄ SO ₄ (1 μg N/g soil)	Soil texture	$Y_{B(N)}(L)$	0.29 - 0.46	(59)

Table 1-2: Microbial growth efficiencies in grassland soils.

Soil Texture	Substrate / Nutrients	Factors	Method	MGE	Ref.
Soddy-podzolic (1.9% orgC)	Glucose (4 mg C/g soil), N, P (C:N:P = 1:10:1/1:30:1)	Substrate Soil type	$V_{\rm P}(S)$	0 80 - 0 95	(7)
Gray forest (2.3% orgC)	Sterilized soil	Substrate, Son type	I <u>R</u> (5)	0.00 0.72	(,)
Umbrisol (28% Clay, 16% totC)	Glucose (300 - 4000 µg C/g soil), C:N:P = 10:1:1	Glucose concentration	$Y_{B}(S)$	0.76 - 0.83	(58)
Sandy loam (29% clay, 5% organic matter)	¹⁴ C-Glucose (0.004 - 400 μg C/g soil)	Glucose concentration	$Y_{B}(L)$	0.47 - 0.66	(63)
Upland taiga, Floodplains	¹⁴ C-Glucose, ¹⁴ C-Phenolics	Substrate, Soil type	$Y_{C}(S)$	0.21 - 0.85	(68)
Loamy (8.7% totC)	¹⁵ NH ₄ ¹⁵ NO ₃	Time	$Y_{B(N)}(L)$	0.15 - 0.60	(25)

Table 1-3: Microbial growth efficiencies in forest soils.

Methods: (S) and (L) indicates incubation duration ≤ 1 day and > 1 day, respectively.

 $Y_{S} = (S_{c}-MR)/S_{c},$

 $Y_B = MP/(MP+MR)$, $Y_{B(N)}$ indicates that MP was calculated from N immobilization

 $Y_C = MB/S_c$

 $Y_R = 1 - (MR/S_c)$

where S_c is the amount of substrate consumed, MR = microbial respiration, and MP = microbial production.

*: No comparisons were made. MGE was measured to calculate microbial biomass turnover and maintenance energy.

One genetic marker that might be used to gauge the efficiency with which bacteria incorporate carbon into biomass is *rrn* operon copy number. Bacteria can have 1 to 15 copies of *rrn* operons per genome (35). Bacteria with high *rrn* operon copy number tend to response faster and are more competitive during high substrate availability. Bacteria with fewer *rrn* operons have ecological strategy characteristics of a K-strategist, with enhanced capability to grow at low substrate concentrations (34). The copy number of *rrn* operons can be predicted from molecular surveys of 16S rDNA. Based on phylogenetic association, we can assign *rrn* operon copy numbers to bacterial groups that respond to environmental factors such as substrate availability. The distribution of *rrn* operons in bacteria and archaea was explored to determine the confidence with which *rrn* operons can be assigned to important phylotypes (39) (Chapter 4).

Microbial Activity in Soil Carbon Models

One of the motivations for understanding how microbes respond to changes in land management practices is the desire to better predict how the practices influence the dynamics of soil organic carbon. Soil nutrient models have been developed to make quantitative predictions that could guide decision-makers on sustainable land management practices (53). Our understanding of the factors that influence carbon transformations must be expressible in mathematical equations to be useful for incorporation into such models.

Microbially driven carbon transformations are typically described using first-order rate kinetics as a function of the carbon concentration (50). Two environmental factors that consistently influence this rate are temperature and moisture, both of which are well documented to correlate with rates of carbon mineralization (1). Growth efficiency is then applied to determine the fraction of the consumed carbon that is transformed into microbial biomass. Due

to a lack of direct measurements of growth efficiency in soil, a fixed value between 0.35 and 0.5 has been commonly assumed in soil carbon models (38, 50, 60). It is currently treated as a global parameter, where the same growth efficiency is used for simulations of all types of terrestrial biomes. This implies that all microbial communities are equally efficient at transforming carbon into microbial biomass. As discussed earlier, growth efficiency is influenced by environmental factors and potentially by changes in community structure. Therefore, understanding regulation of growth efficiency in different biomes is crucial to improve accuracy of site-specific models.

The incorporation of accurate MGE into models will acknowledge the versatility of microbial communities in responding to different substrate type and nutrient status, rather than treating soil microbial communities as a single entity. This is especially important for simulations with short timescales or models with more robust microbial mechanism such as the inclusion of carbon allocation for extracellular enzymes (38, 49). Site-specific MGE measures will also become more important as improved models are able to take into consideration ecosystem changes that result from shifts in microbial communities (46). The application of MGE in SOC models and its significance in understanding microbial-driven soil carbon dynamics are further discussed in Chapter 5.

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CHAPTER 2

MEASURING BACTERIAL GROWTH EFFICIENCY IN SOIL

Introduction

Soil carbon transformations are driven primarily by microbial communities in soil. Bacteria, which can make up to 80% of the microbial biomass, play an important role in decomposing plant-derived organic compounds and consequently in forming soil organic carbon (8). The process of decomposition contributes to soil respiration and immobilizes carbon into microbial biomass, which plays an important role in soil carbon sequestration. The amount of plant-derived carbon that is respired or incorporated into bacterial biomass is determined by growth efficiencies of the bacterial communities.

Bacterial growth efficiency (BGE) is defined as the fraction of the total carbon substrate consumed that is incorporated into new biomass (9). It is calculated from bacterial respiration (BR) and bacterial production (BP). In terrestrial ecosystems, growth efficiency is typically measured for the composite microbial community (which will be referred to as MGE in this dissertation). There are four major methods by which growth efficiency is determined. Each method is based on different assumptions so they are not readily comparable (Table 1)(16).

In aquatic environments, the amount of substrate consumed is typically measured as the depletion of dissolved organic carbon (DOC) while microbial biomass is measured as the accumulation of particulate organic carbon (POC) (10). In soil, organic carbon available to microbes is difficult to quantify and is highly dependent on the extraction method (24). Therefore, the amount of substrate consumed in soil cannot be measured directly. Bacteria are known to have strong interactions with soil particles so the heterogeneous matrix of soil also

makes measuring bacterial biomass difficult (6). Due to these challenges, investigators often use a single radiolabeled compound to determine the use of substrates (Chapter 1, Tables 1-1 to 1-3).

MGE Calculation	Parameters measured	Assumptions
$Y_s = (S_C - MR)/S_C$	Substrate consumed	All substrate consumed that is not respired is
	CO ₂ produced	incorporated into biomass.
		Extracellular compounds are considered as
		incorporated carbon.
$Y_{\rm P} = MP/(MP+MR)$	Microbial biomass	The amount of substrate consumed is equal to
- D	CO ₂ produced	the sum of biomass carbon and respired
		carbon.
		Extracellular compounds are not considered as
		incorporated carbon.
$Y_{C} = MB/S_{C}$	Substrate consumed	Carbon consumed that is not incorporated into
	Microbial biomass	biomass material is respired.
		Extracellular compounds are not considered as
		incorporated carbon.
$Y_{\rm P} = 1 \cdot (MR/S_{\rm T})$	Substrate added	Added carbon that is not respired is
	CO ₂ produced	incorporated into biomass material.
	CO2 produced	Extracellular compounds are considered as
		incorporated carbon.

Table 2-1: Methods for calculating growth efficiency and the corresponding assumptions. $S_C =$ Substrate consumed, MR = microbial respiration, MP = microbial production, $S_T =$ total substrate.

One drawback to using a single radiolabeled compound is that the growth efficiency measured will not be representative of all carbon transformations by the overall bacterial community unless every member is able to transport and metabolize the added substrate. The organic compounds in soil that serve as carbon or energy sources for heterotrophs are a mixture of saccharides, organic acids, amino acids and phenolics (7). The relative abundance of these different compounds can vary with the plant species growing on the soil (29). If the carbon substrate used to measure BGE represents only a small fraction of the total available carbon, the growth efficiency measurement will be a poor measure of overall bacterial activity.

An example of the problems associated with using single substrates is provided by observations using radiolabeled glucose. MGE varies with the concentration of glucose applied

to the soil (4). At low concentrations of substrate MGE is very high - in some cases even higher than the growth efficiency of pure cultures during unconstrained balanced growth (2, 32). Bremen and Kuikman suggested that MGE was overestimated when the amount of substrate provided was too low because it could be assimilated even though the microbes were unable to initiate growth (3). Later studies demonstrated that when carbon is provided at low concentrations it is stored in the cytoplasm (32).

In addition to the affects of varying substrate concentration, the influence of extended incubations on MGE has also been measured. Over a period of 100 days, substrate availability in the soil microcosm decreased, as did the concentrations of other nutrients such as nitrogen and phosphorus. Bacterial community will also change in both physiological state and composition. As a consequence growth efficiency was observed to decrease with incubation time (19). Hill et al. suggest that MGE should be measured shortly after the soil is collected because root exudates such as glucose have extremely short half-lives (20).

It is therefore important to have growth efficiency measurements that are reflective of the nutrient status in the soil rather than the added substrate. This is particularly significant when comparing the efficiencies of microbial communities across different sites. It is also important to distinguish the properties of bacteria and fungi as they contribute differently to soil carbon sequestration and respond differently to environmental factors (26, 35). This chapter introduces a method to measure BGE in soil without the addition of exogenous carbon substrate. It is adapted from bacterial productivity measurements in aquatic ecosystems (27). The method measures BP by incorporation of trace amounts of leucine and simultaneously measures BR by the consumption of oxygen (Figure 2-1).

Materials and Methods

Soil Sampling

Soil samples were collected from the W. K. Kellogg Biological Station Long Term Ecological Research main site (KBS LTER, Hickory Corners, MI) in August 2009 and Spring 2010. The site description and soil type are available at http://kbs.msu.edu. Five soil cores of 2.5 cm diameter were collected from the top 10 cm at the conventional agriculture sites with a corn/soybean/wheat crop rotation (T1), deciduous forests (DF), and native grassland (T8). The crop at the conventional agriculture sites was soybean in 2009 and wheat in 2010. The litter layer in the deciduous forest soil was removed prior to sampling. Soil cores from each experimental site were pooled and brought back to the laboratory on ice. The soil was homogenized with a 4 mm sieve and stored at 12°C until used for experiments. All measurements were made within 48 hours of sampling. Soil pH and moisture were determined prior to the start of an experiment (34).

Soil Incubation

Field moist soil samples were resuspended in 10 mM MES buffer (1:1 w:v). The buffer was adjusted to the same pH as the soil and supplemented with 50 μ g/ml cycloheximide to inhibit fungal growth and 2.3 mM sodium pyrophosphate to assist with dispersion of bacteria. The soil suspension was stirred for 30 minutes at 200 rpm then filtered through 8 layers of cheesecloth followed by a 100 μ m cell strainer (BD, Franklin Lakes, NJ) to remove large soil particles. Unlabeled L-leucine (Sigma-Aldrich, MO) was added to 5 ml of the soil slurry to a final concentration of 2 μ M. A sample of 550 μ l was removed for measuring oxygen consumption prior to addition of radiolabeled leucine. Immediately after ³H-leucine (250 nCi, Sigma-Aldrich, MO) was added, 50 μ l slurry was removed and added to 5 ml scintillation

cocktail (Biosafe II, RPI Corp., Mt. Prospect, IL) to determine total radioactivity. To determine leucine saturation kinetics, the concentration of added leucine was varied with different amounts of unlabeled L-leucine. Final concentrations ranged from 0.3 nM to 110 μ M. The soil slurry was incubated for 30 minutes at 25^oC while shaking at 100 rpm. To determine the linearity of leucine incorporation, soil slurries with 0.66 μ M leucine were sampled over a 1 hour incubation.



Figure 2-1: Workflow of measuring BGE for soil samples using ³H-leucine. TCA: trichloroacetic acid.

When BGE was determined using ¹⁴C-acetate, the soil slurry was incubated in a 60 ml serum bottle with a magnetic stir bar. The serum bottle was sealed and 50 μ l was sampled for determining total radioactivity immediately following the addition of ¹⁴C-acetate. This was a modification of the method developed by Eichorst for measuring BGE in pure cultures (13).
Bacterial Production

250 μ l of the soil slurry spiked with ³H-leucine was collected in chilled 2 ml screw-cap tube containing 100% trichloroacetic acid (TCA, final concentration 5% (v/v)) at time zero (T₀) and after 30-minute incubation (T_{30}) . For measuring the linearity of incorporation the slurry was also sampled 60 minutes after addition of labeled leucine. The acid mixtures were stored at $5^{\circ}C$ for at least 30 minutes to precipitate macromolecules. The method for determining ³H-leucine incorporation rate was adapted from Bååth, E. et al. (1). Briefly, the TCA-precipitated macromolecules were collected by centrifugation at 11,000 g for 15 minutes and washed with 1 ml 5% TCA followed by 80% ethanol. After the last washing step, the pellets were dissolved in 0.4 ml 1M sodium hydroxide and incubated in a 90° C water bath for 1 hour with vortexing every 15 minutes. The dissolved pellets were cooled to room temperature before mixing with scintillation cocktail. All supernatant fractions from a sample were pooled and 1 ml was used to determine the amount of radioactivity that had not been incorporated into biomass. Samples were kept in scintillation cocktail overnight in the dark before quantifying the radioactivity with a Beckman Coulter LS 6000TA Liquid Scintillation Counter (Brea, CA), courtesy of Dr. Yong-Hui Zheng. All counts were corrected for background and quenching. Quenching factors were determined by internal-standard method (30). The average recovery of the radiolabeled ³Hleucine was $85.6\% \pm 1\%$.

The amount of leucine incorporated into biomass was determined as the 3 H-leucine in TCA precipitate at T₃₀ minus 3 H-leucine in TCA precipitate at T₀. The incorporated leucine was

converted into the total amount of protein synthesized and total carbon incorporated using the conversion factors suggested by Simon and Azam (38). All leucine incorporated during the 30 minute incubation was assumed to be biological since a formaldehyde-treated soil slurry had no significant incorporation of leucine into TCA precipitate (data not shown).

When measured using ¹⁴C-acetate incorporation, TCA (final concentration 5% (v/v)) was added after 30 minutes incubation to stop growth and to acidify the slurry so that the CO_2 produced was released into the headspace. The serum bottle was stirred in an ice-bath on a magnetic stir plate to allow simultaneous precipitation of macromolecules. TCA-precipitated samples were collected into chilled 2 ml screw cap tubes after 2 hours of venting with nitrogen gas. The TCA-precipitated slurry was processed as described above. It is assumed that all ¹⁴C label in the TCA-precipitate is carbon incorporated into biomass.

Bacterial Respiration

Oxygen consumption during the 30-minute incubation was determined by monitoring the oxygen concentration of a 0.5 ml sample in an oxygen chamber with a micro-respirometer (Unisense, Denmark). The amount of oxygen consumed was converted to carbon units by assuming a respiratory quotient (RQ) of 1. Basal RQ for bulk soil incubated in a static condition averages 0.78 and does not exceed 1 when incubated for a short period (11). In this experiment the soil slurries were shaking in ambient oxygen condition as they were incubated to promote aerobic heterotrophy as the major metabolic pathway.

Carbon dioxide produced in the soil slurry spiked with 14 C-acetate was released into the headspace of the serum bottle when the slurry was acidified with 5% TCA. The 14 CO₂ was

vented for two hours into three phenethlyamine:methanol (1:1 v/v) base traps arranged in tandem. Triplicate 1 ml trap solution was collected and each added to 5 ml scintillation cocktail to measure 14 CO₂ captured.

Data Analysis

Bacterial production from the incorporation of ³H-leucine into macromolecules and respiration from oxygen consumption were expressed as the mass of carbon and normalized to soil dry weight and per hour (μ g C/g dry soil/hr). BGE was calculated from these two parameters using the formula BGE = BP/(BP+BR). Measurements from soils collected from three experimental sites for each biome were treated as biological replicates except when noted. In the ¹⁴C-acetate method BP and BR were the radioactivity in macromolecule fraction and in base trap, respectively.

Differences between methods and samples were assessed using the Student's t-test. All statistical analyses were performed with R: A Language and Environment for Statistical Computing (39).

Results

Leucine Incorporation

The rate of leucine incorporation increased with leucine concentrations ranging from 0.3 nM to 110 μ M (Figure 2-2a). No saturation of leucine incorporation was observed for the range of concentration tested. However, two different patterns of incorporation were observed. At concentrations below 1 μ M, the amount of leucine incorporated increased linearly with leucine

concentration (slope = $1.23 \pm 0.01 \ \mu g \ C/g \ dry \ soil/hr/\mu M$ leucine). At higher concentrations, the change in leucine incorporation rate per μ M leucine concentration decreased by half (slope = $0.06 \pm 0.004 \ \mu g \ C/g \ dry \ soil/hr/\mu M$ leucine) (Figure 2-2a). Michaelis-Menten kinetics fitted to leucine incorporation rates at concentrations below 5 μ M suggests that saturation of some leucine transporters can be achieved at concentrations above 1 μ M (Figure 2-2b). Leucine incorporation is linear for at least one hour of incubation (Figure 2-3).



Figure 2-2. a) Leucine incorporation rate (μ g C/g soil/hr) by bacterial communities in grassland soil for leucine concentrations between 0.3 nM and 110 μ M. b) Leucine incorporation rate for leucine concentrations below 5 μ M fitted to Michaelis-Menton kinetics (V_{max} = 1.1 μ g C/g soil/hr, Km = 0.97 μ M). Each point represents the average of three experimental replicate with one standard deviation as the error bars. Grassland soil was used in this experiment as the trials performed during method development were tested using grassland soils.

Bacterial Growth Efficiency

BGE was calculated for soil slurries from measurements of leucine incorporated into TCA-precipitable material and oxygen consumption (BGE_H). This method was compared to the method using 14 C-acetate (BGE_C) for soil slurries prepared from crop rotation and forest soils

collected in February and March. BGE_C is on average two-fold higher than BGE_H from the same sample. BGE_C was consistently higher for bacterial communities in forest soils than the communities from the rotation soils (Figure 2-4). The BGE_C of rotation soil bacterial communities increased significantly in March, although still lower than in forest soils. On the other hand, BGE_H was not significantly different between the two biomes or sampling times.



Figure 2-3. Incorporation of leucine over time for grassland soil at KBS LTER when leucine concentration is 660 nM. Each symbol represents average of three biological replicates. The error bar is smaller than the symbols. The linear regression has an r^2 of 0.995.

Discussion

Measuring growth efficiency

Growth efficiency for microbial communities in soil is typically measured using a tracer substrate, such as glucose or acetate (Chapter 1, Tables 1-1 to 1-3). These are substrates common in soil but the total organic carbon available to bacterial soil communities is a much more complicated mixture of saccharides, carboxylic acids, amino acids and the more resistant phenolic compounds (31). Radiolabeled tracer substrate is often favored because it is a direct measure of carbon allocation since no conversion factors are required. Additionally, the use of stable isotopes instead of radioactive compounds for this method can be used to identify bacterial groups that are involved in transforming the particular carbon substrate (18).



Figure 2-4: Bacterial growth efficiency in rotation and forest soils measured using ³H-leucine/oxygen (striped) and ¹⁴C-acetate (solid) in February and March.

Understanding bacterial growth on a single substrate can be important in describing the dynamics of that particular substrate in different environments. For example, forest soils appear to have acetate oxidizers that are more efficient than the ones in the rotation soils (Figure 2-4). However, it is impossible to determine how much a single carbon substrate, when incorporated into bacterial biomass, contributes to overall soil organic carbon stabilization. Our interest is how soil bacteria in different soil types or under different land management influence carbon cycling. Therefore, it was crucial to develop a method that would allow the measurement of growth efficiency on the mixed carbon substrates present in the soils.

Bacterial Production

The use of ³H-leucine as a measure of bacterial production was introduced for aquatic systems by Kirchman et al. and later developed by Simon and Azam (27, 38). It is a sensitive measure of bacterial production because bacteria make protein (and hence, incorporating leucine) throughout cell division. It is also advantageous that protein makes up a large fraction of total cell biomass and this fraction is relatively constant. Therefore leucine incorporation can be reliably converted into biomass production (38). Using protein synthesis as a measure of bacterial production avoids the uncertainty of the molecular fate when carbon is assimilated. Leucine specifically reflects carbon that is incorporated into biomass and not carbon assimilated and stored (as when using glucose). Nguyen and Guckert showed that when MGE is calculated from ¹⁴C-glucose, MGE decreased by 24% when only ¹⁴C incorporated into structural biomass (non-fumigation extractable biomass) is taken into account (32).

The ³H-leucine incorporation method was later modified by Baath et al. to measure production of bacteria extracted from soil (1). The extraction step to prepare the bacteria for leucine incorporation is likely to change the physiology of the bacteria. Recovery of bacterial cells might be variable, depending on the interaction between bacterial cells present and their interactions with soil particles. To reduce potential stress on the bacterial community, we eliminated the centrifugation step of Baath et al. and simply made a soil slurry. This further allows the BP measurement to be reflective of the substrates typically available in the soil sample, rather than exogenously provided substrate.

The drawback of measuring BP using radiolabeled leucine is that incorporation is dependent on leucine concentration. A saturating concentration is required to assure that the BP measured is not limited by leucine. However, this is challenging as no saturating asymptote was observed for the grassland soil with leucine concentrations up to 110 μ M (Figure 2-2). Saturating concentrations for other amino acids such as glycine have been reported to be in the mM range (23). The difficulty in saturating leucine uptake is not surprising because the high diversity of bacteria in soil can include transporters with a wide range of kinetics. The different kinetics observed could be due to changes in the type of leucine transporters that are active. Thus when leucine concentration increases, the high affinity transporters become saturated while the low affinity transporters are activated.

E. coli and *Pseudomonas aeruginosa* have high affinity transporters with Km's ranging from 0.2 to 0.34 μ M (22, 33). Although no complete saturation was observed, biphasic leucine incorporation kinetics indicates that some transporters, such as the high affinity transporters are being saturated. This is consistent with the finding that leucine incorporation rate at low leucine concentrations can be fitted to the Michaelis-Menten function. Biphasic leucine saturation kinetics was previously observed for microbial communities associated with decaying wetland litter and for pure cultures (12, 17). Buesing and Gessner suggested avoiding leucine concentrations in the μ M range as they are more likely to promote uptake by fungi (5). Therefore in our study the concentration of leucine used for BP measurement was 2 μ M. It was selected to saturate the high affinity transporters and minimize assimilation by fungal communities. It was also expected that most bacteria in soil would be expressing the high affinity transporters because amino acids in soil that are available for bacteria are present in extremely low concentrations (21). Additionally, it was important to use lower concentrations to minimize the use of leucine as carbon and energy source preventing the measured BGE from representing bacterial growth on leucine.

To identify appropriate incubation times over which to measure productivity, the kinetics of radiolabeled leucine incorporation were monitored over the course of one hour and shown to increase linearly over this period. However a shorter incubation time of 30 minutes was selected to minimize changes in the composition of the bacterial community (Figure 2-3). This short incubation time also reduces the risk of the community becoming carbon-limited because the carbon made available from homogenizing the soil aggregates can be rapidly decomposed (20). Precipitation of macromolecules using trichloroacetic acid is required to determine the amount of leucine incorporated because up to 50% of the leucine is taken up within minutes of addition but not incorporated into protein (data not shown, (21)). An incubation of 30 minutes is also suitable for measuring oxygen consumption because oxygen was not depleted. Therefore BP and BR measurements could be made simultaneously.

Bacterial Respiration

Bacterial respiration has typically been measured as CO_2 production, but the need for short incubation times (30 minutes) prohibited accurate measures of CO_2 production in the soil slurry. Fortunately recent technological developments in oxygen sensors have made direct measurements of oxygen concentration more practical. The Unisense respirometer is highly sensitive and allows measurements to be made in as short as 10 minutes. The drawback to oxygen consumption as a measure of bacterial respiration is the need to know the respiratory quotient (RQ) to convert the amount of oxygen consumed to the amount of carbon respired. RQ for agriculture or forest soils are found to range between 0.77 and 0.8 (11). It approaches 1.0 only when substrate is added in concentrations higher than 100 μ g C/g dry soil (11). In this study, the amount of water-soluble organic carbon in the slurry ranged between 223 μ g C/g dry soil and 623 μ g C/g dry soil. The disruption of soil aggregates during slurry preparation is expected to increase substrate availability when compared to bulk soil. In addition, the soil slurry was incubated shaking, maintaining a well-aerated condition to promote aerobic respiration. Hence, an RQ = 1 was assumed to be reasonable. If the RQ had been assumed to be 0.77 then there would be approximately 6% underestimation of BGE, which is not a significant change (data not shown).

Bacterial Growth Efficiency

BGE_C and BGE_H were both calculated using the Y_C method (Table 2-1). A high BGE close to the theoretical maximum of 0.8 was obtained when using radiolabeled acetate to measure BGE in the forest soil (Figure 2-1). This is unexpected as acetate has less free energy available than glucose or glycerol. BGE measurements for *E. coli* growing on acetate are approximately half of the BGE when glycerol is supplied as the sole carbon and energy source (data not shown). The correlation between substrate quality and growth efficiency was also observed for soil samples (14). Based on previous short-term soil MGE measurements using radiolabeled glucose, it was expected that BGE_C using acetate would be below 0.5 (Chapter 1, Tables 1-1 to 1-3). The high BGE_C-acetate obtained in this study is consistent with our hypothesis that added substrate will be taken up and stored instead of mineralized if other nutrients are limiting or the conditions do not support growth (3, 32, 36).

On the other hand, the lower BGE obtained using the ³H-leucine/oxygen method is consistent with BGE measurements calculated using ammonium nitrate. Schimel introduced a C:N-dependent method where microbial production is calculated from the immobilization of ammonium nitrate (37). Yet, the BGE_H measurements for KBS LTER soils are at the lower range of Schimel's agriculture soils MGE measurement (Chapter 1, Table 1-1). This is consistent with the prediction that KBS LTER soils are not conducive for efficient growth.

Another reason that BGE_H is lower than BGE_C is that BGE_C does not take into consideration use of substrate by bacteria that are not actively synthesizing new biomass. This subgroup of bacteria does not contribute to carbon sequestration but can still be respiring to generate maintenance energy. It is unclear how much microbial respiration is from such metabolically arrested cells, but a large fraction of the bacterial community in soil is expected to be in a non-growing state (28). The ³H-leucine/oxygen method includes respiration from all bacterial cells in the soil slurry whereas BGE_C only accounts for carbon respired from the added acetate. Respiration from non-acetate derived carbon by metabolically arrested cells could explain some of the difference between BGE_H and BGE_C .

As discussed in Chapter 1, growth efficiency is highly dependent on substrate availability and nutrient status. BGE_H was measured for soil slurries prepared from bulk soil. This means that the BGE measured is more likely to represent the potential growth efficiency with all the substrates and nutrients available in the soil slurry. In the field, carbon availability is sparse and more heterogeneous. A large fraction of soil carbon is found within small aggregates and is not available to majority of the bacterial community *in situ* (25). However, if all the soil samples were treated the same way during slurry preparation, differences in BGE measurements are not due to differences in heterogeneity but are due to different active bacterial communities or environmental factors such as nutrient status.

Conclusion

The use of single radiolabeled substrates is useful for determining the fate of carbon in the substrate or to identify a particular group of decomposers (15, 18). However, it is not a suitable method to measure the overall bacterial growth efficiency in soil. Cross-site comparisons to determine the influence of land management practices require the understanding of the function performed by the composite bacterial communities and in the presence of substrate typically available for growth. Therefore, we suggest that cross-site comparisons of bacterial growth efficiency should be made using the ³H-leucine/oxygen combination introduced here.

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CHAPTER 3

BACTERIAL GROWTH EFFICIENCY IN SOILS UNDER DIFFERENT LAND MANAGEMENT PRACTICES

Introduction

Soil bacteria play a central role in transformations of soil organic carbon (SOC) and nutrient cycling. Carbon inputs, in the form of root exudates and litter, are decomposed and transformed into bacterial biomass. Bacteria produce metabolites and extracellular enzymes that assist with humification and aggregation, both of which increase the protection of soil organic matter from further decomposition (35). Bacterial biomass continues to contribute to the stabilization of soil organic matter even after the cells die, as biomass can serve as a substrate for humification (24). Understanding the involvement of microbes in soil carbon sequestration is becoming more important as there is increasing evidence that biological factors strongly influence the dynamics of dissolved organic carbon (18, 23).

Soil heterotrophic bacteria depend on carbon input from plants as both carbon and energy sources. Therefore transformation of carbon from plants into bacterial biomass is not complete because some of the carbon is respired into the atmosphere as CO₂. When a change in CO₂ production is observed, it could represent a change in the production of plant-derived carbon, the rate of decomposition of plant compounds or a change in growth efficiency. Bacterial growth efficiency (BGE) is a measure of the fraction of consumed carbon that is being incorporated into new biomass (Chapter 2, (9)). It is an informative measure of the physiological state of the bacterial community and its ability to stabilize labile organic carbon. In pure culture studies, BGE was found to be sensitive to both substrate quality and availability, and can rapidly decrease at low substrate concentrations (38). BGE can also be influenced by innate processes of

the bacterial community, which are dictated by the genetic makeup of its constituents (32). Different bacterial species can have different energy requirements for both growth and non-growth purposes, and can use catabolic pathways that yield different amounts of energy.

Even though BGE provides valuable information, measurements in terrestrial ecosystems have been sparse. Comparisons across studies are challenging because many use different methods and assumptions (2, 17, 25, 30). One particularly common complication is that growth efficiency measurements vary with the concentrations of labeled substrates used in the experiment. Additionally, growth efficiency in soil is often measured for the total microbial community consisting of both bacteria and fungi. Yet, it is essential to elucidate the function of these communities separately because bacteria and fungi respond differently in soils under different land managements (21, 28). Fungi are typically assumed to be more efficient than bacteria, but agricultural soils with different fungal:bacterial biomass ratios were found to have similar growth efficiency (40). It is also important to distinguish the role of bacteria and fungi if the objective is to link community dynamics to the changes in function of bacteria and fungi in carbon transformations.

A survey of microbial growth efficiency in terrestrial ecosystems suggests that it is sensitive to temperature, soil type, substrate quality and availability, but not fungal:bacterial ratio (7, 11, 14, 34, 40). However, growth efficiency is still widely assumed to be constant for terrestrial biomes (26, 31). Focusing on bacterial communities, we tested the assumption that BGE is constant for soils under different land managements. Bacterial biomass production and respiration were measured without the addition of exogenous substrate to calculate the potential growth efficiency of bacterial communities in soil collected from a forest and three differently managed croplands at the Kellogg Biological Station Long Term Ecological Research (KBS

LTER) site. The sensitivity of BGE to resource availability was determined by laboratory incubation of soil from tilled, corn/soybean/wheat rotation cropland with different nutrient amendments.

Materials and Methods

Soil Sampling

Soil samples were collected from the W. K. Kellogg Biological Station Long Term Ecological Research main site (KBS LTER, Hickory Corners, MI) in 2010. The site description and soil characteristics are available at http://kbs.msu.edu. Five soil cores of 2.5 cm diameter were collected from the top 10cm at the conventional agriculture sites with corn/soybean/wheat crop rotation (T1), deciduous forests (DF), soybean monoculture cropland (B19) and a cultivated land that was left fallow (B21). The crop at the conventional agriculture sites when the soil was sampled was wheat. The litter layer in the deciduous forest was removed prior to sampling. Soil cores collected from each experimental site were pooled and brought back to lab on ice. The soils were homogenized through a 4mm sieve and stored at 12°C until used for experiments. All measurements were made within 48 hours of soil sampling. Soil pH and moisture were determined prior to the start of experiments (27). Soil samples were also frozen at -80°C for chemical analysis.

Soil Chemistry

Water-soluble dissolved organic carbon (DOC) and total dissolved nitrogen (DN) were extracted using a protocol modified from Jones and Willett (19). Briefly, frozen soil samples were suspended in sterile water (1:2 w/v) supplemented with sodium pyrophosphate. The soil

slurry was shaken at 200 rpm for 30 minutes followed by centrifugation at 8000g for 10 minutes. All steps were performed at 4^oC to reduce decomposition. The supernatant was filtered through 0.45µm syringe filter to remove particulate organic matter, including most bacteria. DOC and DN were measured using a Shimadzu TOC-TN analyzer (Shimadzu, Columbia, MD), courtesy of Dr. Steve Hamilton at KBS.

Bacterial Growth Efficiency

BGE was measured according to the ³H-leucine/oxygen method described in Chapter 2.

Microcosm Experiments

Field-moist soil samples from rotational croplands were mixed with talc that was saturated with buffer or amendment solutions (10 μ l solution/50 mg talc/g soil). The amendment solutions were labile carbon mixture, inorganic nitrogen, carbon and nitrogen mixture, or R2 broth (Difco, Detroit, MI). The labile carbon solution contains a combination of glucose, fructose, sucrose, lactic acid, citric acid, succinic acid, and acetic acid. It is a modification of the artificial root exudate solution used by Baudoin et al. (3). The saccharides provided twice the amount of carbon supplied by the carboxylic acids, providing a total of 250 μ g C/g dry soil. Nitrogen was added at a final concentration of 25 μ g N/g dry soil. The carbon and nitrogen mixture has a C:N ratio of 10. R2 broth was applied at 100 μ l/g dry soil, providing 342 μ g C and 19 μ g N per g of dry soil. The microcosms were incubated in the dark at 25^oC for 24 hours. Soil slurries were prepared from these microcosms and BP and BR measurements were made as

described in Chapter 2. Soils were collected at three different experimental sites of rotation cropland to prepare three microcosms for each treatment.

Data Analysis

The incorporation rate of ³H-leucine into macromolecules was used as a measure of bacterial production (BP), while bacterial respiration (BR) was based on measurement of oxygen consumption rate. Both rate measurements were converted to mass of carbon and normalized to soil dry weight and time of incubation (μ g C/g dry soil/hr). Bacterial Growth Efficiency (BGE) was calculated from these two parameters using the formula BGE = BP/(BP+BR). Soils collected from three different experimental sites for each biome were treated as independent biological replicates of each management practice.

ANOVA was used to assess treatment effects while the effect of seasonality was assessed by repeated measures ANOVA. Pair-wise comparisons between treatments were evaluated by Tukey's HSD. For the treatment effect, only measurements that were performed on the same dates (June, August and September) were included in the analysis. Statistical significance is recognized at p-value ≤ 0.1 . All statistical analyses were performed with R: A Language and Environment for Statistical Computing (37).

Results

BGE in field samples

Bacterial production (BP) and respiration (BR) were measured for soil slurries prepared from soil sampled from four different land managements. Although archaea could be contributing to the measured production and respiration, archaea contributes only 1.4% of the total rRNA sequences in KBS LTER soils (33). Generally, a positive correlation was observed between BR and BP (Fig. 3-1a, $r^2 = 0.20$, p-value < 0.001). However, only 20% of the variation in BR could be explained by variation in BP. Forest and soybean monoculture cropland soils have similar respiration rates but the bacterial community from soybean monoculture soils synthesized more biomass (data not shown).

The measured BP and BR were used to calculate bacterial growth efficiency. BGE varies almost 3-fold, from 0.23 to 0.63. Soils under different land managements have bacterial communities that exhibit different BGE (Figure. 3-1b, ANOVA p-value = 0.001). With a median BGE of 0.52, the bacterial communities in soybean monoculture cropland soils are the most efficient, allocating up to 63% of substrate carbon into biomass. The median BGE is at least 9% more than the other three biomes. Bacterial communities in fallow soils have the lowest bacterial production (0.84 μ g C/g dry soil/hr) but with a median BGE of 0.43. These deciduous forest soils have bacterial communities with a BGE of 0.37, which is below the commonly assumed growth efficiency of 0.45 in soil (26).

BGE in rotation cropland soils is the most variable, ranging from 0.24 to 0.59 (Figure. 3-1b). Some of this variability is due to a seasonal effect (Figure. 3-2, RM-ANOVA p-value < 0.001). All three experimental sites of rotation cropland soils exhibit a similar trend, where two peaks of BGE were observed. The first peak occurred in May, during active wheat growth, and the second peak was observed in September, one and half months after the wheat crop was harvested and the field was left bare. This seasonal variation was not observed for forest soils, which stayed constant throughout the year (Figure. 3-2).



Figure 3-1. a) The linear relationship between bacterial production and respiration for soil samples from four different biomes and at different times of the year. The solid line represents the linear fit, BR = 0.51*BP + 1.19, $r^2 = 0.20$, p-value < 0.001. b) Boxplot showing the variation of BGE in the four different biomes from June through September. Different letter indicates significant difference from post-hoc Tukey HSD test with a p-value ≤ 0.1 (n=9).



Figure 3-2. Seasonal variation of BGE in rotation cropland (dashed line) and deciduous forest (solid line). Each point represents three experimental replicates and error bars are standard error.

BGE in microcosm amendments

To ascertain the influence of substrate availability on BGE, measurements were made of BP and BR for 24-hour microcosms prepared from rotation cropland soil. Carbon amendments of

easily oxidizable saccharides and carboxylic acids commonly found in root exudates did not stimulate BP. Instead, it increased respiration rate almost two-fold, which led to a significant decrease in BGE from 0.49 in the unamended control to 0.40 (Figure 3-3). Nitrogen addition by itself had no influence on BP, BR or BGE (Figure 3-3). When carbon was added in combination with inorganic nitrogen there was a simultaneous increase in both BP and BR such that BGE remained the same as in the unamended control. A microcosm with R2 broth, a rich medium commonly used to isolate heterotrophs from the environment, was the only microcosm that exhibited increased BGE compared to the control (Figure 3-3b).



Figure 3-3. Relative a) Bacterial production, bacterial respiration and b) BGE for microcosms amended with carbon, inorganic nitrogen, carbon and inorganic nitrogen mixture or R2 broth. All measurements are normalized to the unamended microcosms by percentage difference. Asterisks represent significant difference from the unamended microcosm, where '*' has p-value ≤ 0.1 and '**' has p-value ≤ 0.05 (Student's t-test).

Discussion

Using the ³H-leucine/oxygen method, BGE in terrestrial ecosystems varied three-fold, from 0.23 to 0.63 (Figure 3-1). It is acknowledged that the BGE measurements more likely represent potential growth efficiency of the bacterial community than efficiency *in situ* because they were made in a soil slurry instead of bulk soil. In the field, carbon availability is lower and less homogeneous; a large fraction is found within aggregates and is not available to a majority of the bacterial community (20). Slurry preparation will disrupt soil aggregates and allow some of the resources in the bulk soil to become more accessible. However, all the soil samples were treated the same way during slurry preparation and therefore differences in BGE are due to differences either in the bacterial community from the different soil treatments (further discussed in Chapter 4) or in environmental factors between soil samples, such as total available carbon and nutrients.

Measurements of BGE in KBS LTER soils are within a narrower range than in aquatic environments, where BGE ranges from 0.025 to 0.81, (9). All four biomes in this study are rich in carbon compared to oligotrophic lakes with their very dilute concentration of dissolved organic carbon. Even for the relatively low carbon input fallow soils, BGE is maintained in the range of 0.26 to 0.46 (Figure 3-1). In the oligotrophic aquatic environment where substrate availability is low, bacteria express a plethora of catabolic genes to scavenge any substrate as soon as it becomes available. This is the "generalist" approach to surviving in low substrate environments (13). This can be costly because more energy must be available for synthesis of proteins and maintaining an active, responsive state. In soil, substrate diffusion is slower, allowing time for catabolic enzymes to be synthesized only when the substrate is made available. Additionally, a large fraction of bacteria in soil is likely to be in a metabolically arrested state, which requires less maintenance energy than an active cell (22).

More than half of the soil samples analyzed exhibited median growth efficiencies lower than the commonly assumed microbial growth efficiency of 0.41 to 0.45 (42). In fact, all but one sample in the forest soils had BGE less than 0.45. Unless the growth efficiency of the fungal community is much higher than the bacterial community, an assumption of 0.45 overestimates the efficiency of carbon transformation by microbes in forest soil. The fungal community is generally assumed to be more efficient than the bacterial community, due to higher structural biomass in fungi, but there is insufficient evidence to suggest that this is always the case (40). Furthermore, actual BGEs in bulk soil are expected to be lower than the potential BGEs measured in soil slurries in these experiments.

The large variation in BGE across all samples indicates that biomass synthesis and energy synthesis activity are not tightly coupled in soil bacteria. The community is likely to be either energy or nutrient starved, as expected for environmental samples where growth substrates are often limiting. When carbon substrates do become available, the stoichiometry of nutrients might not be ideal for increased productivity but it can still be assimilated and mineralized. This is often observed when high C:N ratio substrates, such as root exudates, are released into the soil (15). The mineralization of carbon without increased biomass synthesis can be explained by maintenance energy requirement or by energy spilling reactions.

Energy spilling reactions are more likely to explain the short-lived increase in respiration observed for soil bacteria flushed with high concentration of carbon substrates. Without nutrients such as nitrogen and phosphorus, the ability to make biomass is still limited. But to maintain an active state, the cells will have to recycle reducing agents such as NADH and hence consume

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oxygen and produce CO_2 (29). This is especially important for those soil bacteria that are unable to store carbon sources (43). On the other hand, a large fraction of bacteria in the soil are in a metabolically arrested state (22). With the exception of spore-formers, bacteria that are in this state still require energy sources for non-growth purposes (maintenance energy) (1, 41). The low growth efficiency for forest soil is in agreement with the stoichiometry of microbial biomass in forest soil. Forest microbial biomass tend to have high C:N and N:P ratio, indicating phosphorus limitation, which will require the highest maintenance energy (8, 39).

The microcosm study with rotation cropland soil suggests that biomass synthesis is not carbon limited because carbon amendment does not increase productivity (Figure 3-3a). In fact, we propose that the C:N ratio is a more important driver of BGE in terrestrial ecosystems than carbon availability, which is a major driver of BGE in aquatic ecosystems (10). The increased BGE observed for soybean monoculture cropland is correlated with increased nitrogen content in soil during growth of soybean (36). When BGE was measured for the rotation cropland during soybean growth, a BGE of 0.46 ± 0.05 was observed. BGE of this magnitude was observed during wheat growth only in May, when it was 0.48 ± 0.02 . This peak of BGE in wheat coincides with two applications of nitrogen fertilizer during the week before the May sample was collected. Our hypothesis is supported by the observation that the seasonal variation in BGE is driven by changes in C:N ratio (Figure 3-4a). The variation in C:N ratio explains 27% of BGE variation in forest and rotation cropland soils (Figure 3-4b).

The lack of response when nitrogen is added individually to microcosms indicates that the bacterial community is not nitrogen limited. However evidence for synergistic effects of nitrogen supplementation and carbon addition was found in the microcosm study. The addition of nitrogen with carbon at a C:N ratio of 10 increases bacterial production by two-fold, even though the same amount of carbon was mineralized as in the microcosm amended with carbon only (Figure 3-3). It appears that the microcosms are nitrogen and carbon co-limited. This means that increased sequestration of carbon into bacterial biomass can be promoted only if increased carbon availability is accompanied by supplementation with other nutrients required for biomass synthesis.



Figure 3-4. a) Seasonal variation of C:N ratio in forest and rotation cropland soils. Each point represents three experimental replicates and error bars represent standard error. b) The linear correlation between C:N ratio and BGE in forest and rotation cropland soils collected in March through September. The solid line represents the linear regression, BGE = -0.04(C:N ratio) + 0.68, r² = 0.27, p-value = 0.001.

Soil bacteria are often described as carbon starved because carbon addition often leads to increased CO_2 production (12). The extra mineralized carbon comes not only from the added substrate but also from older SOC (16). This phenomenon is also known as the priming effect (6). The use of CO_2 production as a measure of bacterial activity does not differentiate between increased growth (activity) and decreased efficiency. Our BGE measurements demonstrate that the increased CO_2 production is not due to increased growth but is instead caused by a decreased efficiency of carbon transformation (Figure 3-3).

A priming effect associated with decreased growth efficiency will eventually lead to loss of soil carbon, as there is no replenishing of the older organic carbon by increasing biomass synthesis. Growth efficiency will decrease if the added carbon stimulates the bacterial community to tap into older soil organic matter for nitrogen or an energy source to synthesize biomass from the added carbon (5). Therefore, carbon addition by itself, either through increased plants per unit area, or by CO_2 fertilization, will not necessarily lead to increased carbon sequestration in soil.

Conclusion

The assessment of BGE in four terrestrial biomes at KBS LTER sites showed that it is not constant. Bacterial communities in soils under different land managements have different growth efficiencies. This work suggests that C:N ratio is the major regulator of BGE in soil. Understanding BGE dynamics can help clarify how soil carbon transformation is being affected by changes in land management practices. Further investigation into carbon availability and nutrient requirements will have to be performed to determine what C:N ratio will support high growth efficiency, promoting bacterial-driven carbon sequestration in soil (4).

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CHAPTER 4

rRNA OPERON COPY NUMBER AS A GENETIC MARKER FOR ECOLOGICAL STRATEGIES

Some of these results have been published in the article: Lee, Z. M. P., C. Bussema, and T. M. Schmidt. 2009. rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. Nucleic Acids Res **37:**D489-D493.

Introduction

Soil harbors a high diversity of bacteria with more than 10,000 species in a gram of soil (35). The structure of bacterial communities in soil has been well characterized using cultureindependent molecular surveys. Molecular analyses have allowed scientists to glimpse into the diversity of bacteria in various environments and to observe how the community responds to perturbations (17). This is especially important because we have yet to culture representative strains from more than 90% of bacterial species in soil (18).

A particular molecule that has been exploited as a phylogenetic marker to identify bacteria in the environment is the 16S rRNA (31). Along with 21 ribosomal proteins, the 16S rRNA makes the 30S small subunit of the ribosome. Due to the central role of ribosomes in protein synthesis, the rRNA gene sequence has regions that are highly conserved interspersed with variable region. A variable region of the rRNA gene can be amplified using primers annealing to the flanking conserved regions. Sequence analysis of the variable region based on databases of known bacterial species, such as Ribosomal Database Project and SILVA, will allow identification of the bacterial genome that harbors the particular sequence (8, 33).

Surveys of genes encoding for central enzyme in specialized functions like denitrification and methane oxidation have allowed the functions of various groups of bacteria to be defined
(23). Soil carbon transformation or CO_2 production on the other hand, is a general function with a high degree of functional redundancy (3). This makes it challenging to link the structure of the bacterial community to general function and it is often found that changes in the function (ie. CO_2 production) is not correlated with shifts in the community composition (15). There is no single gene that can be targeted as a marker for CO_2 production because different bacteria can process substrates using multiple different pathways. Consequently, perturbations of soil microcosms, or survey of the terrestrial ecosystem is often based on community composition determined by 16S rDNA sequence analysis.

One aspect of 16S rDNA surveys that is often overlooked is the presence of multiple genes per genome that encode the rRNAs. The 16S rRNA genes, along with the 23S and 5S rRNA genes, have a unique characteristic relative to other bacterial genes. It can be present from one to 15 copies per genome (21). The multiplicity of rRNA genes has been recognized as a caveat in molecular analyses targeting rRNA genes (19). It is a hindrance in quantifying relative abundance of different sequences. A bacterium with seven copies of 16S rRNA genes per genome will appear to be more abundant than a bacterium with two copies of the gene, even if the two species were present in equal abundance. The copy number effect becomes more influential for environments dominated by bacteria with different rRNA gene copy numbers. However, with increasing characterization of the rRNA operon copy number of ecologically relevant bacteria, their abundance can be better described. This has led to the compilation of rrnDB, an online database cataloging rRNA genes copy number in bacteria and archaea (http://rrndb.mmg.msu.edu/, (22)).

Albeit a concern, rRNA genes copy number is a potential genetic marker for understanding the ecological strategies of bacteria. This chapter explores the distribution of rRNA genes in bacteria and discusses the characteristics of bacteria with different rRNA genes copy number. Using molecular survey of bacterial community in rotation cropland and forest soils at the KBS LTER site, we used rRNA genes copy number to make hypothesis regarding the ecological strategies of the bacterial communities.

Abundance of rrn operons in Bacteria

Ribosomal RNA genes in bacteria are typically arranged in an operon, the *rrn* operon. The first gene is the 16S rDNA (*rrs*), followed by 23S rDNA (*rrl*) and 5S rDNA (*rrf*) with an internal transcribed spacer (ITS) region between *rrs* and *rrl* (6). There are five different groups of ITS, some of which can encode up to four tRNA genes (41). The intragenomic heterogeneity of the ITS region also makes it a suitable target for culture-independent identification when the rDNA heterogeneity does not provide sufficient resolution such as at the subspecies level (43). While 5'-*rrs*-*rrl*-*rrf*-3' is the general operon structure, there are various exceptions. The second most commonly found operon structure is the separation of the *rrs* gene from the *rrl*-*rrf* genes cluster. This structure is common in intracellular bacteria, such as members of the class Rickettsiales, and other host-associated bacteria such as species in the genus Helicobacter (25, 28). Members of the family Brachyspiraceae and Leptospiraceae in the phylum Spirochaetes have three separate promoters for each of the rRNA genes. All of the species with unusual *rrn* operon structure has either one or two sets of rRNA genes.

Bacterial species with one or two copies of *rrn* operon dominate the current catalogue of *rrn* operon copy number (22). Bacteria with eight copies or more only makes up 10.3% of unique

species in the catalog (Figure 4-1). It consists of members of the phylum Firmicutes and class Gammaproteobacteria with only one member from the Betaproteobacteria class (Figure 4-2). Two well-defined genera that belong exclusively in this group are Shewanella and Vibrio. There are currently only two species with 15 *rrn* operons – *Clostridium paradoxum* and *Photobacterium profundum* (34, 45). Most genera have members with the same *rrn* operon copy number or differ by one. However, some genera such as Lactobacillus and Clostridium can have a wider range of *rrn* operon copy number (Table 4-1). It is important to note that the *rrn* operon copy number distribution is highly biased towards bacteria with fully sequenced genomes. It does not necessarily mean that the domain Bacteria is dominated with low *rrn* operon copy number members.

Genus	rrs copies
Lactobacillus	1-9
Geobacter, Gluconacetobacter, Pelobacter	2-4
Bifidobacterium	2-5
Aquifex	2/6
Rhodobacter	3-4
Ruegeria	3/5
Corynebacterium, Ralstonia	3-6
Clostridium	3-15
Burkholderia	4-6
Bacteroides, Pseudomonas, Streptococcus	4-7
Acinetobacter, Methylobacterium	5-7
Pseudoalteromonas	5/9
Bacillus	7-14
Alkaliphilus, Desulfotomaculum	8-10
Shewanella	8-12
Paenibacillus, Vibrio	8-13

Table 4-1: Genera with more than two rrs copy number.

Archaeal genomes have a smaller range of rrn operon copy number, with only 1 to 4 copies per genome. Members with one rrn operon dominate the catalogue, constituting 60% of the archaea in the rrnDB (22). Archaea with more than one rrn operon are all from the phylum

Euryarchaeota. Archaeal genomes are also often found to have the 5'-*rrs*-*rrl*-3' genes cluster encoded separately from *rrf*. This unusual operon is found in all known *Thermoplasma* genomes.



Figure 4-1: Distribution of 16S rRNA gene copy number in bacteria. The analysis was performed on 652 unique bacterial species (gray bars).

In molecular surveys of 16S rRNA gene, two sequences with a distance of more than 3% difference are often defined to be in separate operational taxonomic units (OTUs). This is often seen as equivalent to different species although the exact definition of species is still debatable (39). When multiple *rrn* operons are present in a genome, it is common to find some sequence differences (1). However, intragenomic heterogeneity of the 16S rRNA gene is typically below 1% (1). The highest level of divergence within a bacterial genome was reported for *Thermomonospora chromogena* at 6.6% (Table 4-2). The archaeon *Halosimplex carlsbadense* has operons with the largest sequence divergence at 7.4% (Table 4-2, (5)). Acinas et al. raised a concern where intragenomic heterogeneity could lead to overestimation in diversity of microbial communities (1). With the exceptions of bacteria and archaea listed in Table 4-2, diversity

measures based on the well-accepted OTU cutoff of 3% will not be influenced by intragenomic heterogeneity (7).

Organism	<i>rrn</i> operon (#)	Divergence (%)	Ref.
Bacteria			
Thermobispora bispora	4	6.4%	(47)
Thermomonospora chromogena	6	6.6%*	(48)
Thermoanaerobacter tengcongensis	4	6.5%	(1)
Thermoanaerobacter pseudethanolicus	4	3.7%*	
Desulfotomaculum kuznetsovii	Unknown	8.3%	(44)
Desulfitobacter hafniense	6	4.8%*	
Photobacterium profundum	15	5.1%	(45)
Archaea			
Haloarcula marismortui	2	5.6%*	(29)
Halosimplex carlsbadense	2	7.4%*	(5)
Natrinema sp. XA3-1	4	5.4%*	(5)

Table 4-2: Sequence divergence of the *rrs* gene and *rrn* operon copy number of bacteria with intragenomic heterogeneity of more than 3%. *Sequence divergence was calculated from neighbor-joining distance matrix produced from the Arb software. The maximum divergence is reported.

Ecological strategies of bacteria with different rrn operon copy number

Ecological strategies of organisms were originally characterized as either *r*- or K-selected by MacArthur and Wilson, based on a set of traits observed in organisms colonizing different stages of succession (26). Andrews and Harris then adopted the *r*/K concept for microbial ecology, proposing that bacteria can be characterized along an *r*/K spectrum based on growth phenotype (4). Bacteria that are *r*-selected have high maximum specific growth rate (μ_{max}) while K-selected bacteria are more competitive in low nutrient environments, utilizing resources more efficiently for biomass synthesis (4). To determine where along the *r*/K spectrum a bacterial species will be placed, the growth characteristics of the bacteria must be determined. This means that a representative of the species must be cultivable.



Figure 4-2: The relative abundance of different phyla for each 16S rRNA gene copy number. For the interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

The combination of genome analysis and bacterial physiology found that high growth rate in bacteria is attributed to increased gene dosage. This can be achieved by having multiple copies per genome or positioning of the gene near the origin of replication (10). Increased numbers of *rrn* operons allow for more ribosome to be synthesized simultaneously and hence, more proteins can be synthesized per unit time when the environmental conditions permit fast growth. In an enzymatic reaction, both enzyme and substrate can be limiting factors. Increasing the amount of ribosomes (the enzyme) can only influence protein synthesis rate if it is accompanied by increased availability of tRNA (the substrate). There is a significant positive correlation between rRNA gene copy number and the abundance of tRNA genes in the genome (Figure 4-3)(12, 22). This observation provides evidence for a selection pressure to maintain high *rrn* operon copy number.

It is generally observed that bacteria with more *rrn* operons have a higher growth rate than bacteria with fewer *rrn* operons, supporting Couturier and Rocha's observation of a

correlation between growth rate and gene dosage (10). Dethlefsen and Schmidt showed that in addition to gene dosage, bacteria with more *rrn* operons have ribosomes that are faster than ribosomes in bacteria with fewer *rrn* operons (12). In the environment, where resource availability is more variable, the benefit of having high *rrn* operon copy number is to be able to respond faster to favorable growth conditions. Klappenbach et al. found that soil bacteria that form early colonies on agar plate have on average, three-fold more *rrn* operons than late-forming colonies (20). In the same study, the addition of a herbicide changed the relative abundance of herbicide degraders where herbicide amended soils select for high *rrn* operon copy number degraders (20). Using *E. coli* as the model organism, others have provided further evidence that multiple *rrn* operons is crucial for fast response time (9, 40).



Figure 4-3: Correlation between 16S rRNA gene copy number and tRNA genes in bacterial genomes. The data are gathered from 867 bacterial genomes. The solid line represents the linear fit, y = 31.8x + 6.3, $R^2 = 0.78$, p-value < 0.001.

A faster response time will allow bacteria with high *rrn* operon copy number to be more competitive in environments experiencing high nutrient pulses. Members of the genera

Shewanella and Vibrio with eight or more *rrn* operons are often found to be in nutrient rich environments. Shewanella are versatile in their respiratory capability and are often found in association with fermenters, exploiting almost any fermentative carbon substrates and electron acceptors made available to them (30). The genus Vibrio consists of one of the fastest growing bacteria, *Vibrio natriegens*, with a doubling time of less than 10 minutes (2, 13).

However, there is a trade-off to maintaining multiple *rrn* operons. Although ribosome synthesis is a highly regulated process, the *rrn* operon promoters are constitutive promoters (24, 37). This means that a basal amount of rRNA will always be synthesized. When conditions become unfavorable for growth, the cost of rRNA synthesis will become a burden to the cells. Some high *rrn* operon copy number bacteria such as *E. coli* adapt to nutrient scarcity by dimerizing the ribosomes (46). This inactivates the ribosomes so that protein synthesis can be downregulated. Under this condition, bacteria with fewer *rrn* operons will become more competitive. Model oligotrophic organisms such as caulobacters and acidobacteria spp. that are abundant in low nutrient environments have one or two *rrn* operon(s) (14, 32).

Based on the correlations of *rrn* operon copy number and various physiological characteristics of bacteria, it is suggested that *rrn* operon copy number is a potential indicator of the ecological strategy of the bacteria, including the rate and efficiency of substrate utilization. Bacteria with high *rrn* operon copy number resemble *r*-strategists, where it is selected for high specific growth rate, with ribosomes that have higher translational power, respond quickly to increased resource availability and are more competitive in environments that experience frequent pulses of nutrient (Table 4-3). On the other hand, bacteria with few *rrn* operons can be considered as K-strategists that are more competitive in low nutrient environments and more efficient at resource allocation to biomass (Table 4-3).

7 15

rRNA gene copy number per genome

1

Few <i>rrn</i> operons Less tRNA encoding genes (11)	Genome	Multiple <i>rrn</i> operons More tRNA encoding genes
Low translational power (11)	Translation	High translational power
Low ribosome drop-off frequency	machinery	High ribosome drop-off frequency
Low growth rates Form late colonies on agar plates (20) Late responders to nutrient input (20) Oligotrophs Low K _{min}	Physiology	High growth rates Form early colonies on agar plates Early responders to nutrient Copiotrophs High K_{min}
High BGE^{0}		Low BGE
K-selected phenotype Widely distributed		<i>r</i> -selected phenotype
Highly abundant in environment with constant low nutrient supply	Ecology	More abundant in environment with frequent pulse of high nutrient.

Table 4-3. Characteristics of the lifestyle of bacteria that are correlated with its *rrn* operon copy number. The descriptions in bold have yet to be tested. ^aThis characteristic is further discussed in Appendix A. ^bThis has been shown by Roller, B. in the Schmidt lab.

The *rrn* operon copy number of environmental 16S rDNA sequences can be determined based on phylogenetic association to close relatives with known *rrn* operon copy number. The *rrn* operon copy numbers can be found in the *rrn*DB database (22). This will allow hypotheses to be made regarding the growth efficiency of the bacteria because we can infer ecological strategies based on *rrn* operon copy number. It can also assist in setting up a microbial community component in ecosystem models (27).

Bacterial communities in soil



Figure 4-4: Relative abundance of bacterial phyla in 16S rDNA libraries constructed from rotation cropland and forest soils in September 2009. Each bar represents average relative abundance from two replicate plots and error bar is one standard deviation. '*' represents significant differences from Student's t-test with p-value < 0.05.

The bacterial communities in forest and rotation cropland soils from the KBS LTER main site were characterized by surveying the 16S rRNA gene sequences. At the phylum level, bacterial communities from forest and rotation cropland have similar composition. Only the phyla Verrucomicrobia, Planctomycetes and TM6 exhibited significant differences, where they are more abundant in forest soil (Figure 4-4). The phyla Proteobacteria, Acidobacteria and Verrucomicrobia describe more than 70% of the bacterial communities. This is not surprising as these three phyla are often found in soil. When compared to other terrestrial biomes, the KBS LTER forest and rotation soils have more Verrucomicrobia and Acidobacteria than grassland soils (42).

Sequence analysis at a finer level of resolution, the order level, revealed more differences between the two bacterial communities (data not shown). In addition to orders in the phyla Verrucomicrobia and Planctomyces, higher relative abundance of the order Rhizobiales was also observed in the forest soil. On the other hand, Sphingomonadales was found to be more abundant in the rotation cropland. The differences between forest and rotation soil bacterial communities at the order level can be observed even when the analysis was performed on the 20 most abundant OTUs (cutoff = 0.03). An exception is orders within the phylum Acidobacteria. There are more sequences clustering with Acidobacteria from the 20 most abundant OTUs at the order level in the rotation cropland soil. These OTUs make up more than 10% of the total bacterial 16S rDNA sequences from rotation soil (Figure 4-5).

To analyze the ecological strategies of the bacterial community from these two biomes, *rrn* operon copy number was assigned to each OTU based on taxonomic classification at the order level. The predicted average *rrn* operon copy number for bacterial communities from both sites is 2.0 ± 0.05 . This is slightly lower than the rRNA gene:single copy housekeeping gene ratio calculated from metagenomic library of the forest and rotation soils (Forest = 2.6 ± 0.1 , Rotation = 3.1 ± 0.3 , Teal, Gomez and Schmidt, 2011, manuscript in preparation). This discrepancy is due to several reasons. Metagenomic libraries have a lower coverage than libraries generated from pyrosequencing of 16S rDNA amplicons. It represents only up to 85 genomes, an extremely small fraction of the overall bacterial community, even if it is only compared to the 20 most abundant OTUs (Teal et al., 2011, manuscript in preparation). Additionally, *rrn* operon

copy number cannot be assigned to 1% of the 20 most abundant OTUs due to lack of information from close relatives (classified as Bacteria in Figure 4-5).



Figure 4-5: Relative abundance of the 20 most abundant OTUs in forest and rotation cropland based on the order level of the OTU. The possible *rrn* operon copy number is provided in brackets. ^P, represents taxonomic classification above the order level. '*' represents significant difference based on Student's t-test with p-value < 0.05.

However, the similarity of average *rrn* operon copy number in the forest and rotation soil suggests that the dominant bacteria have K-selected phenotypes. This suggests low concentrations of resources in both the forest and rotation soils, selecting for bacteria that are more efficient at resource use. Based on the average *rrn* operon copy number, it is predicted that the bacterial community in forest and rotation soils have similar growth efficiency when exposed to the same growth conditions. Therefore, variation in growth efficiency between the forest and rotation soil is more likely to be driven by environmental factors.

Most soils are often seen as a nutrient limiting environment but not all bacterial communities are similar in terms of *rrn* operon copy number. A survey of 71 soil samples from

biomes with different carbon content found that acidobacteria are more prevalent in low resource availability biomes while the abundance of copiotrophic Bacteroidetes and Betaproteobacteria is positively correlated with increased resource availability (16). Furthermore, it is often observed that when low molecular weight substrates such as glucose and citric acid are added to soil, Acidobacteria becomes outcompeted by fast-growing bacteria with high *rrn* operon copy number such as Burkholderia spp. (4 - 6 copies) and Flavobacterium spp. (6 copies) (15). On the other hand, the abundance of members of the phylum Actinobacteria does not correlate with carbon availability. This phylum has a wider range of *rrn* operon copy numbers, with the Corynebacterium and Streptomyces having six copies and the cellulose degraders Cellulomonas having only two *rrn* operons. These observations provide further support for characterizing bacteria based on ecological strategies using *rrn* operon copy number.

Conclusion

The application of molecular surveys has provided considerable insight into the structure of bacterial communities in soil. Efforts to link general function such as CO₂ production to community composition has been difficult because all metabolically active heterotrophs in soil produce CO₂, but more specialized functions like methane oxidation can been linked to the diversity of methane-oxidizing bacteria. The capacity to identify ecological strategies of bacteria in soil could help accelerate our understanding on how aboveground activity influences bacterial activity. We suggest that *rrn* operon copy number is a suitable candidate as an indicator for the ecological strategies of bacteria. Bacteria with high *rrn* operon copy number tend to have the phenotype of an *r*-strategist while low *rrn* operon copy number bacteria will be more competitive in low nutrient environments. Therefore, the inclusion of *rrn* operon copy number classification

in molecular analyses can provide information regarding the ecology of the bacterial community and make predictions on the influence of community composition on bacterial growth efficiency.

Materials and Methods

Soil Sampling

Soil samples were collected from the W. K. Kellogg Biological Station Long Term Ecological Research main site (KBS LTER, Hickory Corners, MI) in September 2009. The site description and soil type is available at http://kbs.msu.edu. Five soil cores of 2.5 cm diameter were collected from the top 10cm at the rotation cropland sites with corn/soybean/wheat crop rotation and deciduous forests. The rotation cropland was planted with soybean at the time of collection. The litter layer in the deciduous forest soil was removed prior to sampling. Soil cores from each site were pooled, sieved and flash frozen with liquid nitrogen in the field, and transported back to the laboratory on ice. Soils were stored at -80^oC upon arrival at the laboratory.

DNA Extraction

DNA extraction from two replicate plots of each biome was performed by Dr. Tracy Teal according to the protocol in Teal et al. (Teal et al., manuscript in preparation). Briefly, DNA was extracted using a direct soil extraction method followed by cesium-chloride gradient purification (36). DNA samples were sent to the Josephine Bay Paul Center in Comparative Molecular Biology and Evolution at Marine Biological Laboratory, Woods Hole, MA., for amplification and sequencing of the V6 region in the rDNA sequence.

Data Analysis

The OTU assignments were downloaded from the Visualization and Analysis of Microbial Population Structures (VAMPS) project at the Josephine Bay Paul Center (38). *rrn* operon copy number was assigned based on the most specific taxonomy level of the sequence. If the *rrn* operon copy number for that taxonomy level is not available, the next broader taxonomy level is used to obtain a range of *rrn* operon copy number. The copy number for the *rrn* operon is obtained from the *rrn*DB database (22).

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CHAPTER 5

SIGNIFICANCE AND FUTURE DIRECTIONS

Bacterial growth efficiency (BGE) is a measure of how bacteria allocate resources for biomass or energy synthesis. Growth efficiency is a central parameter in soil organic carbon (SOC) models that simulate microbial-driven organic carbon transformations. Soil carbon models are important for determining sustainable land management practices. One such model is the DAYCENT model, the daily version of the CENTURY model. DAYCENT is a biogeochemistry model that simulates plant-soil nutrient cycling, specifically carbon, nitrogen, phosphorus and sulfur dynamics in terrestrial ecosystems (12, 14). In the model, growth efficiency is applied to determine the fractionation of carbon at various steps of microbially driven carbon transformations. Microbial growth efficiency (MGE) is currently treated as a global parameter, where a single value is used for simulating carbon cycling and carbon dioxide flux in various biomes, from forests to grasslands and conventional agricultural soils (2, 15). In the DAYCENT model, most growth efficiencies are currently fixed at 0.45. This means that for every microbial-driven carbon transformation, 55% of the transformed carbon is lost into the atmosphere as CO₂, while the remaining 45% is incorporated into microbial biomass (14). By using a constant growth efficiency value, the DAYCENT model assumes that organic compounds with different stabilities are converted with equal efficiency into microbial biomass, and that microbes from different biomes have equivalent growth efficiencies.

In Chapter 3, it was shown that bacterial communities, which can constitute up to 80% of total microbial biomass in soils, in land under different management practices have different growth efficiencies (Chapter 3, (3)). BGE ranged from 0.23 to 0.63, with soybean monoculture

cropland soil having the most efficient bacterial communities (Chapter 3). More than 70% of the soil samples have BGE less than 0.45. This means that a growth efficiency of 0.45 will be overestimating the efficiency of carbon immobilized into microbial biomass when simulating some of the biomes at KBS LTER. Schimel and Weintraub introduced a model with a growth efficiency value of 0.35 to take into account the allocation of substrate for extracellular enzyme production (17). This model would be a better representation of microbially driven carbon dynamics in KBS LTER soils.

A sensitivity analysis was performed using the DAYCENT model to determine if the variation in BGE observed for field samples was sufficient to change soil carbon dynamics. An executable version of the DAYCENT model was obtained from Cindy Keough at the National Resource Ecology Laboratory at Colorado State University. A mock ecosystem was simulated using the site-specific parameters for Kellogg Biological Station and vegetation parameters for a temperate deciduous forest. Both input parameters were obtained from the Vegetation Ecosystem Modeling and Analysis Project (VEMAP) simulation, which is available through Oak Ridge National Laboratory Distributed Active Archive Center (http://daac.ornl.gov/MODELS/guides/century vemap.html, retrieved on February 12, 2011, (10)). The weather input for DAYCENT model was obtained from the National Weather Service Station at KBS (retrieved from KBS LTER Data Catalog on October 31, 2010). The mock ecosystem was simulated for 1990 years to initiate soil organic carbon pools and plant biomass, and to allow the system to reach equilibrium. The output from the initial simulation was then used to run a simulation for 70 years with different global values for microbial growth efficiency.

There are ten fixed growth efficiency parameters in the DAYCENT soil organic carbon submodel (Figure 5-1). By default, seven of these growth efficiency parameters are fixed at a value of 0.45. Surface structural carbon is composed of crop residue with high lignin content. Lignin is more recalcitrant to microbial degradation, thus the high lignin component of crop residue enters the slow carbon pool without being completely decomposed by the microbial community. The fungal community, which is often assumed to be more efficient than the bacterial community, is primarily thought to be the major decomposer of surface structural carbon (6, 14). Hence, the growth efficiency applied for decomposition of surface structural carbon is 0.55. The incorporation of surface microbial carbon into slow carbon is thought to have the highest efficiency at 0.6. On the other hand, bacterial community is responsible for decomposition of soil active carbon into the slow carbon pool is a function of soil texture, where the efficiency is lowest in soil with high sand content (Figure 5-1, (14)).

In the sensitivity analysis, the seven growth efficiency parameters originally fixed at 0.45 were varied. The active carbon pool was most sensitive to changes in growth efficiency. This carbon pool consists of root exudates, deposition from dead root cells, active microbial biomass and microbial metabolites. A 22% decrease in the carbon pool was observed when growth efficiency was decreased from 0.45 to 0.35 (Figure 5-2a). This was expected since growth efficiency is a direct measure of the capability of the microbial community to incorporate resources into biomass material. Additionally, the decrease in the active carbon pool was sufficient to influence the rate of soil passive carbon accumulation (Figure 5-2b). When the default growth efficiency (0.45) was used in the simulation, the mock ecosystem accumulated passive carbon at a rate of 0.35 $g/m^2/year$. A decrease of growth efficiency by 5% stabilizes passive carbon (0.4, Figure 5-2b). A further decrease to 0.35 led to a loss in passive carbon (0.35, Figure 5-2b).



Figure 5-1: Soil organic carbon submodel in the DAYCENT ecosystem model. Ovals represent the application of growth efficiency in carbon transformations. Blue ovals represent growth efficiency values that were adjusted in the sensitivity analysis. This figure is adapted from the CENTURY User's Manual (13).

Passive carbon formation is a combination of the biological, chemical and physical transformations of carbon. Decreased growth efficiency will reduce the biologically mediated carbon transformations, but the reduction in active carbon pool will decrease the amount of substrate (microbial biomass and extracellular products) available for chemical and physical transformations (9). Stable carbon is often found to have more microbially derived carbon (4, 7, 8). Therefore, to supply more microbially derived carbon for stable carbon formation, it is important to practice land management that will support the growth of microbial communities with high growth efficiency.



Figure 5-2. Sensitivity of the active carbon pool (a) and the annual passive carbon pool (b) to variations in growth efficiency. The active carbon pool at different growth efficiencies is presented as a percentage relative to the active carbon pool when growth efficiency is 0.45.

The sensitivity of ecosystem models to changes in growth efficiency emphasizes the importance of using site-specific values of growth efficiency. This is crucial when the model is used to make predictions about how changes in land management practices could influence SOC dynamics (16). However, to effectively apply growth efficiency in biogeochemistry models, we must better understand how growth efficiency is regulated in the field. Focusing on bacterial growth efficiency, the work from this dissertation presents a framework for how BGE can be

influenced by climate change, soil edaphic properties and land management practices (Figure 5-3).



Figure 5-3. Framework relating changes in climate, land management practices and edaphic factors on BGE. The dotted arrows represent processes that can influence BGE.

The two major sources of perturbation in terrestrial ecosystems are climate change and land management practices. These environmental factors can influence the function of bacterial communities in soil, where the function in this context is defined as soil carbon transformation. Bacteria respond to changes in environmental factors as soon as immediately after the perturbation to several hours or days, where the response is typically measured as CO_2 production (11). The response of bacteria to perturbation is also influenced by edaphic factors (20). Some of the major factors that have been shown to exert a change in function are moisture, litter type, and resource availability (19).

Microbial-driven soil carbon transformations are often measured as CO_2 production. While CO_2 production is a good indicator of microbial activity, it is not a good measure of the efficiency at which carbon is transformed into microbial biomass. For example, we found no correlation between soil CO₂ flux and BGE (Figure 5-4). Field CO₂ flux is a combination of root respiration and microbial respiration. Microbial CO₂ production can constitute up to 50% of soil CO₂ efflux (5). However, there are two possible explanations for changes in microbial CO₂ production. First, it can represent an overall change in bacterial growth. This can be measured as change in decomposition rate, either because more bacteria become active or the new condition allows for increased decomposition rate. Change in CO₂ production can also be caused by changes in energy expenditure for biomass synthesis. BGE can distinguish between these two scenarios. If the change in CO₂ production is due to the first reason, one will observe no difference in BGE, while the second situation will lead to a change in BGE. In laboratory incubation, a response was observed as soon as 24 hours after incubation (Chapter 3). It is this immediate response of bacterial communities to changes in aboveground activity that leads to within site variation in BGE (Figure 5-3: short-term response).

Over multiple generations, the changes in the reproduction of individual populations will lead to changes in community structure. This will occur if the perturbation has differential effect on the community members. If the perturbation is not removed, bacteria that can survive better in the new environment will subsequently increase in abundance. If the change in community composition leads to change in ecological strategy, it is expected that growth efficiency will be affected (Chapter 4). This change in growth efficiency is mediated by the potential of the new bacterial community to efficiently allocate substrate for growth. This potential is dictated by the genetic capability of the community, which will influence the type of metabolic pathways used, energy required for cellular maintenance, investment of energy for production of extracellular enzymes to access substrates, efficiency of translational machineries and various energy expenditure processes (18).



Figure 5-4. Seasonal variation of CO_2 flux (a, b) and BGE (c, d) in the conventional agriculture (a, c) and deciduous forest (b, d) sites. Each point represents the average of three experimental replicates and the error bars are standard error. Flux data was obtained from the KBS LTER dataset (retrieved on March 21, 2011).

In conclusion, I have shown that BGE in the terrestrial ecosystem is not static and can be influenced by climate, edaphic properties and land management practices via two pathways. The short-term response is mediated by changes in the function of bacterial communities while the delayed effect is due to changes in community composition. The variability of BGE in the terrestrial ecosystem and sensitivity of carbon models to changes in BGE highlights the need to apply site-specific BGE in SOC models. Future research on the growth efficiency of bacterial

communities in soil should be focused on a wider range of biomes. Attention should be placed on biomes that have resource availability with different C:N:P stoichiometry. Research on terrestrial BGE variation should be conducted in conjunction with the assessment of bacterial communities and soil properties. This will allow variation in BGE to be incorporated into SOC models, where it is a function of community composition and environmental factors.

Finally, the hypothesis that BGE is a suitable indicator of biological carbon sequestration in soil should continue to be tested. The contribution of this dissertation work is consistent with the goal put forth by the scientific community to better understand how changes in aboveground activity influence the stability of soil organic carbon (1).

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APPENDICES

APPENDIX A

STREPTOMYCIN-RESISTANT E. COLI MUTANT EXHIBITS INCREASED BACTERIAL GROWTH EFFICIENCY

Introduction

Protein synthesis, also known as translation, is one of the most expensive cellular processes. It is catalyzed by ribosome, a ribonucleoprotein complex. The catalytic center of the ribosome consists primarily of rRNA while ribosomal proteins play a structural role to increase the efficiency and to stabilize the structure of the ribosome (10). One such protein that plays an important role in determining the efficiency of translation is ribosomal protein S12. It is encoded by the gene *rpsL* and interacts with 16S rRNA as part of the 30S small subunit. Ribosomal protein S12 is a central ribosomal protein in assisting polypeptide elongation and is involved in determining translational accuracy (9).

Some mutation in *rpsL* in *E. coli* can render the bacteria resistant to streptomycin. One very well studied mutation is a point mutation in codon 42 that changes a lysine codon to asparagine. This mutation does not only renders the mutant streptomycin resistant; it also causes the ribosome to be slower at peptide elongation. The decreased elongation rate is correlated with decreased growth rate and increased fidelity (2). Additionally, the *rpsL* mutant also exhibits increased ribosome drop-off frequency (5).

Ribosome drop-off is a phenomenon where the peptidyl-tRNA dissociates from the translational machinery prior to reaching the stop codon. When compared to other translational errors such as missense substitutions and frameshift errors, ribosome drop-off contributes most significantly to inefficient translation (5). Ribosome drop-off event results in formation of free peptidyl-tRNA that arrests protein synthesis if not hydrolyzed by peptidyl-tRNA hydrolase (3).

In wild type *E. coli* K12 strains, ribosome drop-off occurs at a rate of once in 4000 codons (8). During the translation of β-galactosidase mRNA, the 1:4000 codons frequency is equivalent to one drop-off occurrence for every four times translation is initiated. The significance of drop-off occurrence is also supported by the findings that peptidyl-tRNA hydrolase is essential for growth in *E. coli* and *B. subtilis* (7, 12). *E. coli rpsL* mutant with high fidelity ribosomes was shown to have a drop-off frequency of once every two translation initiation (5).

During exponential growth, protein synthesis can consume up to 60% of cellular ATP (17). The ATP spent on making truncated polypeptides is considered wasteful because the ATP is not invested in synthesizing proteins for building biomass. This means that to synthesize the same amount of biomass, the *rpsL* mutant will require more ATP. Increased demand for ATP due to increased ribosome drop-off is expected to decreased bacterial growth efficiency. It is expected that the mutant will allocate less carbon substrate assimilated for biomass because it is mainly used for energy synthesis.

Here I tested the hypothesis that ribosome drop-off frequency is correlated with BGE. I hypothesized that the high fidelity mutant with lower translational yield will need more energy for biomass synthesis. A theoretical calculation predicts that the high fidelity mutant will have a 10% decrease in BGE when compared to wild type. This hypothesis was tested by measuring ribosome drop-off frequency and BGE for *E. coli* K12 wild type and an *rpsL*(K42N) mutant.

Materials and Methods

Strains and growth characterization

The wild-type and streptomycin resistant mutant for ribosome drop-off assay were *E. coli* XAC and *E. coli* SmR obtained from Dr. Albert Dahlberg (11). It is the same strains that were

used in Dong and Kurland's study on ribosome drop-off (5). Cultures were maintained in LB medium, which was also used to grow cultures for recombineering procedures. Ribosome drop-off assay was performed in M9 minimal medium supplemented with 19 amino acids (no methionine) and the carbon source is 0.4% glycerol (M9-Gly54) (8). Another *E. coli* strain used in this study is *E. coli* MG1655 provided by Dr. Robert Britton at Michigan State University. Growth characterization was made in M9 minimal medium and the carbon sources are 0.2% glucose, 0.2% glycerol or 0.4% acetate. *E. coli* mutant rpsL6 was constructed by recombineering as described below, by introducing a single point mutation.

Plasmid construction

All plasmids were constructed by restriction digestion and ligation reactions. The *E. coli* specific plasmid carrying the gene *ylc* (pYLC), which encodes the reporter protein YLC (a chimeric protein from the fusion of enhanced yellow fluorescent protein (EYFP), β-galactosidase and enhanced cyan fluorescent protein (ECFP)) and pYC were constructed by Drs. Uri Levine and Mike Weigand (Figure A-1). Briefly, the gene encoding EYFP was amplified from pEYFP with primers containing HindIII and BamHI restriction sites and cloned into the multiple cloning sites (MCS) upstream of gene encoding ECFP in pECFP (BD Biosciences, Palo Alto, CA). This construction is name pYC. The *lacZ* gene from pSV-β-galactosidase control vector (Promega, Madison, WI) was amplified using primers engineered with BamHI and AgeI restriction sites. Plasmid pYC was digested with restriction enzymes BamHI and AgeI to insert the *lacZ* amplicon between *eyfp* and *ecfp*. This final product is the pYLC plasmid. In both pYC and pYLC plasmids, the genes *yc* and *ylc* are expressed under the control of *lac* promoter, which can be induced by
isopropyl-ß-D-1-thiogalactopyranoside (IPTG). The negative control plasmid, pBla, was constructed by digested pECFP with StuI and PvuII followed by blunt-end ligation.



Figure A-1. Plasmid pYLC. *bla*: ampicillin resistance gene, *ori*: origin of replication, Plac: *lac* promoter, *eyfp*: yellow fluorescent protein gene, *lacZ*: β-galactosidase gene, and *ecfp*: cyan fluorescent protein gene.

Ribosome drop-off

Ribosome drop-off assay requires quantification of independent expression of two reporter proteins, YC and YLC. YC is a fusion of the enhanced yellow fluorescent protein and enhanced cyan fluorescent protein, abbreviated EYFP:ECFP while the YLC chimeric protein has a β-galactosidase inserted between the two fluorescent proteins. Non-specific fluorescence was measured from a culture carrying pBla plasmid, which is the background plasmid of pYC and pYLC.

Overnight cultures of *E. coli* XAC and *E. coli* SmR expressing pBla, pYC and pYLC were inoculated into M9 medium supplemented with 18 amino acids and 100 μ g/ml ampicillin, and 0.4% glycerol as carbon source to an OD₆₀₀ of 0.2 and induced with IPTG to a final concentration of 2 mM. Following induction, OD₆₀₀ and relative fluorescence units (RFU) were measured at every increment of 0.1 OD₆₀₀ until OD₆₀₀ reaches 0.8. Optical density was measured using Spectronic 20D+ while fluorescence were measured using Spectramax M5 spectrofluorometer at excitation 435 nm and emission 475 nm with a cutoff filter at 455 nm for ECFP expression, and excitation 520 nm and emission 575 nm with a cutoff filter at 550 nm for EYFP expression.

The background fluorescence from pBla was substracted from pYC and pYLC expression to correct for any non-specific fluorescence. The corrected fluorescence signals were then divided by the corresponding OD_{600} to normalize to biomass. The ratio of ECFP expression to EYFP expression for each chimeric protein was calculated as RFU(C)/OD divided by RFU(Y)/OD. Ribosome processivity, which is completion of translation, is expressed as,

$$R = \frac{\text{average C/Y ratio from YLC}}{\text{average C/Y ratio from YC}}$$

An R value equals to 1 means that the ribosome always complete translation. If drop-off occurs, there will be less ECFP from YLC than YC and hence, R will be less than 1. Drop-off frequency can be calculated as 1-R.

Construction of K42N mutant by recombineering

Plasmid pSIM6 carrying phage genes required for recombination was obtained from Dr. Robert Britton at Michigan State University. It was introduced into *E. coli* MG1655 by

electroporation according to the protocol from Molecular Cloning (4, 16). Successful transformants were selected by plating on LB agar plates supplemented with 100 µg/ml ampicilin. The transformants were confirmed by PCR amplification of the ori region in plasmid pSIM6 using SD3 (5'-CTGAAAGCACAGCGGAGATGTGTATAAGAGACAGCTGGACAG TAAGA-3')-SD4 (5'-TTGTATGGAACAACGAGATGTGTATAAGAGACAGCTGACGGGT TTTG-3') primer set (4). The expression of recombination genes in the transformants was induced for 15 minutes at 42°C. The induced cells were then made electrocompetent after induction by washing with ice-cold deionized water according to the protocol in Current Protocols in Molecular Biology (18). An 80 bp oligo, RPSL3 (5'-ACG AAC ACG GCA TAC TTT ACG CAG CGC GGA GTT CGG TTT GTT AGG AGT GGT AGT ATA TAC ACG AGT ACA TAC GCC ACG TT-3', corresponding to lagging strand that complements position 208 to 287 in the *rpsL* gene was introduced into the electrocompetent cells expressing recombination genes by electroporation. The electroporated cells were grown in SOC medium for 2 hours at 32°C. Selection of positive recombinants was made by plating the culture on LB agar plate supplemented with 100 µg/ml streptomycin. The pSIM6 plasmid was cured by growing the recombinants at 37°C followed by selection of colonies that do not result in products when amplified with SD3-SD4 primer set. The base pair change in *rpsL* gene was confirmed by sequencing product from PCR amplification with RPSL1-RPSL2 primer set (1).

BGE measurements using ¹⁴C-labeled substrate

BGE measurements for all strains were made in M9 medium with glycerol as the carbon source and supplements were as described in the figures. The strains were grown in 750 ml side-

arm flasks with magnetic stir bars, at 37°C and shaking at 200 rpm. Optical density at 600 nm for each culture was monitored with a Thermo Spectronic 20D+. The cultures were added with uniformly labeled ¹⁴C-glycerol (0.25 µCi, Sigma-Aldrich, St. Louis, MO) at early log-phase, when OD_{600} is approximately 0.1. Immediately after ¹⁴C-glycerol addition, the flasks were sealed and triplicates of 100 µl sample were removed into 5 ml scintillate (Biosafe II, RPI) to determine total label added. The culture was incubated for two doublings and then added with 5% trichloroacetic acid (TCA) to stop growth and to acidify the culture to release any CO₂ produced into the headspace. The headspace was vented with nitrogen gas into three phenethylamine:methanol (1:1 v/v) base trap arranged in tandem for two hours. During these two hours, the acidified culture was placed on ice while stirring to promote precipitation of macromolecules. At the end of the two hours, triplicate of 1 ml samples were collected from the base traps into 5 ml scintillate to measure the activity of ¹⁴C-CO₂. This was the measurement for bacterial respiration (BR). Bacterial production (BP) was measured as the amount of ${}^{14}C$ activity in the TCA precipitated fraction, which was collected by filtration through 0.7 µm glass fiber filter (Whatman GF/F, Piscataway, NJ). Bacterial growth efficiency (BGE) was calculated from BP and BR using the formula BGE = BP/(BP+BR).

Results

Chimeric protein YLC was developed as a reporter protein for quantification of ribosome drop-off. It is a fusion of enhanced yellow fluorescent protein (EYFP), ß-galactosidase and enhanced cyan fluorescent protein (ECFP). Ribosome drop-off will result in a ECFP:EYFP ratio

of less than 1 with the assumption that drop-off occurring prior to the completion of EYFP mRNA is negligible. To correct for differential expression between different strains of bacteria, the ECFP:EYFP ratio from YLC expression was compared to the ratio from YC expression. It is expected that more drop-off will occur during the translation of the longer YLC mRNA and hence, less ECFP will be synthesized from YLC mRNA translation than from YC mRNA translation.

Throughout exponential phase, R in both wild type and streptomycin resistant mutant, strain SmR, exhibit a constant processivity (Figure A-2). The SmR strain has significantly lower ribosome processivity than the wild type strain (Table A-1). This means that the mutant has a higher ribosome drop-off frequency because it completes translation less often than the wild type strain. This result is consistent with Dong and Kurland's measurement for the same strains of *E. coli* (5).



Figure A-2. Ribosome drop-off frequency for wild type (WT) and SmR mutant (SmR) in M9-Gly54 medium during log-phase growth. R-value is calculated as ECFP:EYFP expression from pYLC plasmid/ECFP:EYFP expression for pYC plasmid when induced with 2 mM IPTG. Each point represents the average of three measurements with the standard error.

Growth efficiencies of wild type and SmR mutant were measured in the same medium, M9 medium supplemented with 19 amino acids and 0.4% glycerol as the carbon source (M9-

Gly54). Bacterial growth efficiency was measured using ¹⁴C-glycerol. Unexpectedly, no difference in BGE was detected between the wild type and SmR mutant strains (Figure A-3). However, the amount of glycerol consumed in both wild type and SmR mutant cultures were only 23 and 38%, respectively (data not shown). This indicates that the bacteria are using amino acids as the major carbon and energy source. Therefore, BGE measurement using ¹⁴C-glycerol in this medium is not a suitable representation of the growth efficiencies for these two strains.

BGE measurement using ¹⁴C-glycerol was repeated in M9 medium with 0.2% glycerol as carbon source and supplemented with only proline and arginine. Both strains are auxotrophic for proline and arginine therefore these two amino acids could not be eliminated from the growth medium. In this medium, the mutant was observed to be more efficient than the wild type strain (Figure A-3). BGE measured using ³H-leucine/oxygen consumption method in the same medium yield the same observation, where the mutant is more efficient than the wild type strain (Figure A-3).

Strain	C/Y of pYLC	C/Y of pYC	R value
E. coli XAC	0.796 (0.021)	1.136 (0.041)	0.701 (0.009)
			0.75
<i>E. coli</i> SmR	0.692 (0.033)	1.257 (0.051)	0.550 (0.011)
			0.53

Table A-1. The R value for *E. coli* XAC and *E. coli* SmR strains carrying plasmid pYC or pYLC and their R values. The values shown are the averages of three independent experiments with standard deviations in parentheses. Values in bold are values reported by Dong and Kurland (5).

The capability of streptomycin resistant mutant to be more efficient at resource allocation for growth was confirmed with a different strain of *E. coli*, *E. coli* MG1655 and an rpsL mutant. The mutant, rpsL6-K42N, was constructed by introducing a base pair change into the *rpsL* gene by recombineering. The mutant is resistant to the antibiotic streptomycin and has increased doubling time compared to wild-type strain when grown on glucose as a carbon source (Figure A-4). The doubling time for wild-type increases as the carbon source is changed from glucose to glycerol or acetate. In M9-acetate, the wild type strain had a doubling time four times longer than growth in glucose while the rpsL6-K42N mutant only increased doubling time by 2.8-fold. This resulted in the mutant growing at a faster rate than the wild type strain in M9-acetate (Figure A-4).



Figure A-3. Bacterial growth efficiency of *E. coli* wild-type and streptomycin resistant strains in three different conditions. GlyAA: M9 medium supplemented with 19 amino acids and 0.4% glycerol. GlyPA: M9 medium supplemented with 0.4 mM proline and arginine, and 0.2% glycerol. BGE in GlyPA medium was measured using two different methods; ¹⁴C-glycerol and ³H-leucine/oxygen. The ³H-leucine/oxygen measurement was provided by Roller, B. from Schmidt lab. Each bar represents the average of two replicates with standard deviation as error bars.

Similar to the observations for *E. coli* XAC and *E. coli* SmR, the streptomycin resistant mutant, rpsL6-K42N, is more efficient. It allocates at least 5% more carbon into biomass synthesis when compared to the wild-type strain (Figure A-5). The distinction is more evident when BGE was measured using ³H-leucine/oxygen consumption, which is a more sensitive measure of carbon allocation specifically into biomass.



Figure A-4. Relative doubling time of *E. coli* MG1655 wild-type and rpsL mutant strain (rpsL6-K42N) in M9 minimal medium with 0.2% glucose, 0.2% glycerol or 0.4% acetate as carbon source. The doubling time was normalized to growth of wild-type strain in M9-Glucose. Each bar represents average of three biological replicates and the error bar represents standard error.



Figure A-5. Relative BGE of *E. coli* MG1655 and rpsL mutant in M9 minimal medium with 0.2% glycerol as carbon source. BGE was measured with two different methods and normalized to the WT strain for each method. Each bar represents average of two replicates and the error bar represents standard deviation.

Discussion

Streptomycin resistant mutant with a K42N mutation in ribosomal protein S12 have increased ribosome drop-off frequency when compared to wild-type strain. This is consistent with Dong and Kurland's measurement for these two strains of *E. coli* (5). Premature dissociation of ribosome leads to the formation of truncated proteins that may not be properly folded into its functional conformation. Therefore, it is expected that the mutant will require more ATP per unit functional protein. The high cost of protein synthesis is expected to decrease growth efficiency of the mutant. Contrary to the theoretical expectation, the streptomycin resistant mutant exhibit increased BGE in all the growth conditions tested except when the medium was supplemented with 19 amino acids. Therefore, I was unable to provide evidence that ribosome drop-off can lead to a decrease in growth efficiency.

An alternative explanation for the increased growth efficiency exhibited by the mutant is that missense error, which is the incorporation of the non-cognate tRNA, is more costly than the synthesis of truncated polypeptide. Missense error occurs at a rate of 1 in 1000 to 10000 codons (14) but can increase during nutrient starvation (13). It is hypothesized that missense error can sequester the ClpX protease away from hydrolyzing sigma factor 38 (6). Sigma factor 38, also known as RpoS, is a sigma factor that is being induced during carbon starvation. It is important for stress protection and the mechanism is by arresting metabolic activity while upregulating stress protective enzymes such as enzymes involved in oxidative stress. In the streptomycin resistant mutant, the absence of aberrant proteins due to low missense error allows ClpX protease to hydrolyze RpoS, even when the cells have entered stationary phase (6). This allows the cells to continue incorporating substrate into biomass while in the wild-type strain, increased missense

errors induced an RpoS-dependent growth retardation, allocating more resources to synthesizing protective enzymes.

It was previously reported in *Salmonella enterica*, that K42N mutants had a higher growth rate in medium with poor carbon source (15). This increased growth rate is correlated with decreased RpoS in the cells compared to the wild type strain. In this study, we suggest that the increased growth rate observed in poor medium is due to increased growth efficiency. When grown in the presence of 19 amino acids, the culture does not experience amino acid starvation until it reaches stationary phase, which will not be detected because BGE was measured during exponential growth. However, when majority of the amino acids are removed, the wild type strain may experience amino acid starvation due to inefficient translation.

The dependency of RpoS stability on ClpX protease activity suggests that missense error in wild-type strain is more costly than ribosome drop-off. Therefore, we hypothesized that the increased growth efficiency exhibited by the mutant is due to increased translational accuracy.

Future Directions

To determine if the mutant is truly more efficient at protein synthesis or the increased BGE is due to decreased missense error, we first have to measure the missense error rate for the wild-type and mutant strains under the growth conditions that BGE was measured. BGE measurements and missense error rates must also be measured in the same medium with and without amino acid supplement to determine if the correlation between growth efficiency and ribosome fidelity is dependent on amino acid requirement.

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