FEMS Microbiology Ecology, Volume 93, Issue 10, 1 October 2017, fix114
DOI:10.1093/femsec/fix114

# Bacterial strategies along nutrient and time gradients, revealed by metagenomic analysis of laboratory microcosms.

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**Key words**

Metagenome; Ecological strategy; Ecological succession; r/K-strategy; Copiotrophy; Oligotrophy

**Abstract**

There is considerable interest in the functional basis of ecological strategies amongst bacteria. We used laboratory microcosms based on culturing of elutant from soil, to study the effects of varying initial nutrient concentration, and time succession, on the community metagenome. We found a distinct set of nutrient related or time related changes in the functional metagenome. For example a high nutrient (copiotrophic) strategy was associated with greater abundance of genes related to cell division and cell cycle, while a low nutrient (oligotrophic) strategy had greater abundance of genes related to carbohydrate metabolism and virulence, disease and defense. We also found time related changes in the functional metagenome, revealing a distinct ‘r’ related strategy with greater abundance of genes related to regulation and cell signaling, and a ‘K’ strategy rich in motility and chemotaxis related genes. These different gene-based strategies may help to explain how so many bacterial OTUs coexist in nature, and the functional principles dominating natural communities. In terms of diversity, both the OTU richness and the richness of species assignment of functional genes showed linear correlations with functional gene richness, supporting the hypothesis that greater taxonomic diversity is associated with greater functional diversity, with possible implications for ecosystem stability.

## Introduction

One of the major aims of ecology is to be able to characterize the adaptations which determine where and when each type of organism occurs in nature (Ricklefs and Miller 1999). In the ecology of larger organisms, certain combinations of features or behaviors occur predictably in the context of certain environments and niches, and are known as strategies (Grime 1977; Southwood 1977; Westoby *et al.* 2002). In plant and animal ecology, among the best characterized and most widely recognized of these are the 'r' and 'K' strategies (MacArthur and Wilson 1967). r strategists are described as living in temporarily resource-rich environments, with the potential to grow and mature rapidly, and produce large numbers of young quickly, with poor ability to withstand sustained competition or predation (Pianka 1970). K strategists are described as having the opposite of these characteristics, being adapted to environments where resource supply rates are lower, population densities are higher and competition between individuals and between species is intense. r and K strategists have often been described in relation to ecological succession, with r strategists being abundant in early successional stages and K strategists prevailing in late successional stages (Odum 1969).

In microbial ecology, there has been little explicit discussion of r or K strategies. Instead, attention has concentrated on the two opposing microbial strategies of 'oligotrophy' (specialization to low nutrient conditions) and 'copiotrophy' (specialization to high nutrient conditions) (Koch 2001). There has only been one concerted attempt to define the differences between these two strategies at the inferred functional level of cell physiology and genome functions (Lauro *et al.* 2009). Lauro *et al.*'s study (2009), while a valuable step forward and the inspiration for the present study, involved selected examples of fully sequenced marine

bacterial species and leaves open the need for broader testing in other environments, and using other selection criteria.

There has been little discussion in microbial ecology of the possible similarities and differences between the copiotrophy-oligotrophy axis and the r – K axis of strategies. It seems, however, that copiotrophy-oligotrophy is thought of in a static sense of steady conditions of greater or lesser *resource supply* (since population growth rate and stability is not generally discussed in the context of this axis). The r-K axis as generally discussed in ecology is considered as part of a *time succession* in which a community develops from early conditions of sparse populations and ready availability of resources, to later conditions of sustained crowding and intense competition for resources.

Here we attempted an approach which may help to elucidate the functional strategies which exist in the bacterial world. Whereas field-based observations can accurately incorporate the true complexity of nature, experimental microcosms can go some way towards reducing the huge number of environmental factors that vary simultaneously, and thus give further useful perspectives (Jiang and Patel 2008; Luckinbill 1979). It is true that in a culture based system, there is the inevitable limitation that only a subset of the species present in nature will grow in the system, and that it can never precisely replicate the complexity of the natural world. Nevertheless, the precise control of initial nutrient content, temperature, etc. in culture systems may be useful in elucidating general patterns that might easily be hidden within the vast complexity of natural ecological systems.

Here, we tested for the existence of different strategies in relation to nutrient and incubation time by combining precisely controlled conditions, with an assessment of combined genome capabilities. In this study, the distinctions ‘copiotrophy vs oligotrophy’,

and ‘r-strategy vs k-strategy’ have been used as a relative form. For example, a function related to copiotrophy has been defined as a functional gene for which relative abundances increase along with the increase of initial nutrient concentration. We predicted that the taxonomic assignment of functional genes and functional gene structure of the community would differ in relation to initial nutrient concentration and incubation time.

We tested the following predictions in relation to ecological strategies:

*A) What are the functional characteristics of oliogotrophy vs copiotrophy amongst bacteria*?

Hypothesis A-1) *Cell division and cell cycle related genes will be more abundant under copiotrophic conditions*. It is clear that amongst plants and corals, those growing in conditions of higher rates of nutrient supply (and light supply, for photosynthesizers) are innately able to grow faster and have higher potential rates of tissue growth and extension, and more rapid life cycle completion to reproduction (Grime 1979; Huston 1994). We hypothesized that, as with larger organisms, at high nutrient concentrations bacterial cells are able to acquire enough nutrient for their basic metabolism, and will use supplementary nutrients for cellular growth/reproduction.

Hypothesis A-2) *Under copiotrophic conditions, genes that are themselves related to promoting gene expression will be relatively more abundant.* In this case, we based our prediction on the microbial literature in relation to copiotrophy. For example, rRNA gene copy number has commonly been used as an indicator of copiotrophy (Condon *et al.* 1995; Fegatella *et al.* 1998; Klappenbach *et al.* 2000; Lauro *et al.* 2009). Our intention here was to test this hypothesis under the more precisely controlled conditions of a nutrient gradient in a

culture system.

Hypothesis A-3) *Nutrient accumulator genes will be more abundant under oligotrophic conditions*. Lower nutrient conditions will require greater numbers of genes associated with accumulating nutrients. Stress tolerator plants and lichens that grow under nutrient-poor conditions are known to be very effective accumulators of nutrients (Grime 1979), and by extension we predicted empirical gene function evidence for similar behavior by bacteria.

Hypothesis A-4) *Dormancy and sporulation related genes will be relatively more abundant in copiotrophic conditions.* Here we were testing for a functional gene abundance difference already predicted by Fierer *et al.* (2007). Since copiotrophs cannot grow in nutrient poor conditions, they are expected to survive in a dormant stage until conditions improve (Fierer *et al.* 2007).

Hypothesis A-5) *Functional gene abundance patterns will parallel those found by Lauro et al. (2009).* Using a very different approach, based on classification of fully sequenced selected bacterial genomes Lauro *et al.* (2009) compared species defined as either oliogotrophic or copiotrophic strategists. Using this approach they arrived at an assemblage of functional genes which may define the copiotrophic strategy. We aimed to test the generality of the pattern found by Lauro *et al.* (2009), by using a different approach – a nutrient gradient in a controlled culture system.

*B) What are the functional characteristics of r vs K selected bacteria?*

Hypothesis B-1) *Cell division related genes will be more abundant in the early successional stage*. Cell division is an aspect of growth, particularly in the prokaryotic world. Typically in

the ecology of larger organisms, early successional species grow fast and give many offspring, rapidly expanding their biomass (Bazzaz 1979; Connell and Slatyer 1977; Odum 1969). Thus we expected this pattern to hold true in bacterial succession, revealing itself in greater relative abundance of genes related to rapid growth/reproduction.

Hypothesis B-2) *Genes related to cell-cell interactions will be more abundant in the later successional stages*. It is generally agreed in ecology that later successional ecosystems have more intense and species specific mutualistic and antagonistic interactions (Morriën *et al.* 2017; Odum 1969). We predicted similar trends towards intensity of (either positive or negative) organismic interactions in the late successional stage in the soil bacterial systems we were studying.

Hypothesis B-3) *Genes related to cell motility will be more abundant in the early successional stage.* The 'r' selected plant species that thrive in early successional stages generally have high seed dispersal ability (Huston and Smith 1987; Peroni 1994). Cell motility, which functionally fulfils the same role as the seed dispersal of 'r' selected plants, is important for a bacterial population to reach newly created open patches or spaces of environment that are relatively free of competition. Thus, genes for cell motility will be more abundant in early successional microbes.

C) How does functional gene diversity relate to nutrient concentration and incubation time?

*Hypothesis C: Functional gene diversity will show a humpback curve against initial nutrient concentration or incubation time, resembling the pattern found in OTU diversity in the same experimental system (Song et al. 2016).*

In an earlier paper on this same experimental system, we found a 'humpbacked' pattern of bacterial OTU diversity in relation to nutrient concentration, and also time (Song *et al.* 2016). We anticipated that functional gene diversity would parallel OTU diversity, because different taxa have different genetically based capabilities, and coexistence of different taxa in itself will require separate ecological niches, which are achieved partly by a range of different cellular and metabolic functions. This taps into a wider debate of whether functional diversity at the community level is necessarily a correlate of taxonomic diversity (Griffiths and Philippot 2013; Huston 1997; McCann 2000; Tilman *et al.* 1997).

## Materials and Methods

### *Sample preparation*

As described in Song *et al.* (2016), plates with different nutrient concentrations were prepared. Tryptic Soy Broth was used as a nutrient source, and agar was used as a solidifying agent. Agar culture medium was prepared with original (nutrient A group), 10-fold diluted (nutrient B group), 100-fold diluted (nutrient C group), and 10,000-fold diluted (nutrient E group) Triptic Soy Broth (Difco) concentrations. In the medium, cycloheximide (100μg/ml) was added to inhibit fungal growth. Sterile cellophane film was overlaid on each culture plate to facilitate cell harvest.

Garden soil (0-5 c.m. depth) was collected from an overgrown flowerbed on the campus of Seoul National University (Song *et al.*, 2016). 20 grams of soil was eluted to 190ml of 0.85% NaCl. The sample underwent 10 min of blending followed by 30 min of incubation in a shaking incubator at 24 ℃ at the speed of 250 r.p.m. We diluted soil solution

$10^{-2}$ times, which was the concentration that had the optimal density of derived colonies (Song *et al.*, 2016). We let soil particles settle for 6 hours before plating while the water was kept chilled to 4℃ to prevent community change. We carefully transferred supernatant into a new tube and 100 $\mu\ell$ of supernatant was spread on each prepared culture plate. Plates were incubated at 25℃ for different lengths of time (Day 2, 7, 28, 56, and 84) before final sampling. Plates that showed any fungal colonies on examination under a low power light microscope were discarded (absence of fungi in the remaining sampled plates was later confirmed from the analysis of metagenomes, see below, which consist overwhelmingly of bacterial DNA). 5 plates were pooled together and treated as one replicate. A total of 300 plates (4 nutrient concentrations * 5 incubation time * 3 replicates for each * 5 plates for each replicate) were used for further analysis. DNA from the final samples was extracted using Genomic DNA purification kit (Promega, Madison, Wisconsin, USA) following the manufacturer's guidelines. Extracted DNA was stored at -20℃ for further analysis.

*Sequencing and sequence processing*

DNA samples were sequenced using Illumina Hiseq2000 platform at Celemics (Celemics, Inc., Seoul, Korea). Sequences were uploaded to the Metagenomics Rapid Annotation using Subsystem Technology (MG-RAST) online server version 4.0 for taxonomic and functional annotation (Meyer *et al.* 2008) under the project name "Effect of nutrient concentration and successional stage on structure and function of culturable bacteria" (project ID, 16343). The Refseq database (Pruitt *et al.* 2007) was chosen for taxonomic annotation of functional genes and the SEED database (Overbeek *et al.* 2005) and the Clusters of Orthologous Groups (COG)

database (Tatusov *et al.* 2003) were used for functional annotation. Sequences were annotated with default setting (maximum e-value cutoff of $10^{-5}$, minimum % identity cutoff of 60%, and minimum alignment length cutoff as 15 bp). Even though more than 99.5% of assigned sequence reads were from bacteria, we filtered out non-bacterial sequences for the statistical analysis.

*Chemical analysis of culture material*

Dissolved organic carbon (DOC) and dissolved total nitrogen (DTN) left in the culture material after harvest was measured to check how the nutrient treatment changed over time. Nutrient A and C group, incubation times 2, 28 and 84 weeks were selected for the analysis. Culture material, which had been kept after harvest at -20℃ was melted at room temperature and sent to NICEM (Korea) for chemical analysis. Samples were filtered with hydrophilic polyethersulfone filter and hydrophilic polypropylene filter (Supor®-450 47mm 0.45㎛).

DOC was measured using a total organic carbon analyzer (TOC-L, SHIMADZU, Japan) and DTN was measured with an AutoAnalyzer (SYNCA, BLTEC, Japan) following standard methods provided by the American Public Health Association (APHA), the American Water Works Association (AWWA), and the Water Environment Federation (WEF) (Rice *et al.* 2012).

*Statistical analysis*

To calculate taxonomic diversity, species level assignments of functional genes from the

metagenomes were used, and for functional diversity Subsystem Level 3 data were used. To normalize the number of sequences in each sample, sequences were subsampled based on the number of least abundant sequence reads among all the samples (1,749,521 for species level assignment of functional genes and 530,466 for Level 3 function). Rarefaction curves were drawn with subsampled sequence data. To calculate taxonomic and functional distance between each sample, the number of metagenome sequence assigned at species level and Subsystem Level 3 in each group was square-root transformed and Bray-Curtis dissimilarity was calculated. To test the influence of initial nutrient concentration and incubation time to the bacterial community structure, permutational multivariate analysis of variance (PERMANOVA) test were performed with 999 permutation using ‘adonis’ function in R package ‘vegan’.

To study the gene abundance differences of bacteria in relation to initial nutrient concentration and incubation time, partial Spearman correlation of initial nutrient concentration (or incubation time) and relative abundance of Subsystem Level 1 and Level 2 functional gene categories, controlling for incubation time (or initial nutrient concentration, and vice versa) was calculated using ‘pcor.test’ in R software ‘ppcor’ package. Partial correlation was used since we aimed to discern the nutrient effect and time effect independently and Spearman rank correlation was used since it does not depend on normality or linearity. Relative abundance of genes in each sample was calculated dividing the number of sequences assigned to each category with the sum of sequences successfully annotated to the gene database.

**Results**

*Chemical analysis of culture material*

As shown in Table S1, dissolved organic carbon (DOC) decreased over time, in both the nutrient A and nutrient C treatments which we analysed. However, the DOC of nutrient C treatment never exceeded that of nutrient A, revealing the continuing legacy of initial conditions in terms of nutrient loading of the culture medium. Dissolved total nitrogen (DTN) decreased over time in nutrient C group, but not in the nutrient A group. The scale of the difference between nutrient A and C group was much greater in the case of DTN.

*Taxonomic and functional composition of communities*

The total number of sequences per sample obtained after quality control ranged from 1,962,970 to 4,641,543. The proportion of proteins with no known function and functional genes not assignable to known genera does not decline with decreasing nutrient concentration (Fig. S1-A and B), but declines with incubation time (Fig. S2-A and B). 16S amplicon based results show a similar pattern, unclassified genera being more abundant in later successional stage (Fig. S1 C and Fig. S2 C). The rarefaction curve of species level assignment of functional genes and Level 3 function had reached its saturation point in all samples (Fig. S3-S4). The composition of species level assignment of functional genes and functional profile showed a predictable pattern with progression in either of two distinct directions related to nutrient gradient and incubation time (Fig. 1). The permutational multivariate analysis of variance (PERMANOVA) result shows that nutrient concentration, incubation time, and the interaction between nutrient concentration and incubation time all had significant influence in relation to community structure, both in terms of the composition of species level assignment of functional genes and composition, and the functional gene composition (Table S2 and

Table S3).

*Functional gene categories correlating with initial nutrient concentration*

Table 1 shows the Subsystem Level 1 and Level 2 genes that have strong (rho>0.5 or rho<-0.5) correlation with nutrient concentration when controlling for incubation time. Only the selected Level 2 genes that follow the same (whether positive or negative) pattern with the Level 1 category are shown. Partial correlation results of "Clustering-based subsystem" and "Miscellaneous" were not included in the result section and were not discussed because most of them are putative genes, or genes for which functions are unclear. For Level 1 genes, we also drew a heatmap with normalized relative abundance data (Fig. S5).

As in Table 1, cell division and cell cycle related genes were more abundant at high nutrient concentrations. Also, genes related to RNA metabolism, especially RNA processing and modification related genes, were relatively more abundant at higher nutrient concentrations. Dormancy and sporulation related genes, especially, spore DNA protection related genes, were more abundant in copiotrophic conditions. Many different kinds of amino acid related genes such as genes related to arginine, urea cycle, polyamines, glutamine, glutamate, aspartate, asparagine, lysine, threonine, methionine, and cysteine were also relatively more abundant at higher nutrient concentrations.

By contrast, genes related to energy acquisition (e.g. carbohydrate) or usage (e.g. respiration) were abundant in the low nutrient treatments. Carbohydrate metabolism related genes had negative correlations with initial nutrient concentration. This was not restricted to one type of carbohydrate, but a broad range of molecules including aminosugars, di- and

oligosaccharides, monosaccharides, and polysaccharides. Genes related to protein metabolism, respiration and photosynthesis (although photosynthesis itself would not have been directly selected for, as the cultures were kept in the dark) which include genes related to electron transfer reaction were also abundant in oligotrophic condition. Having high abundance of genes related to virulence, disease, interference competition and defense, especially the genes providing resistance to antibiotics and toxic compounds, was also one of the noticeable characteristics of more the oligotrophic/low nutrient treatments.

We also attempted to annotate sequences based on the COG gene database to compare our results with those of Lauro *et al*. (2009) (Table 2). Note that COG2852 gene was not found in our metagenomes (this gene was also minimally present in Lauro *et al*'s (2009) result), and were thus excluded. When we set the rho cutoff value higher than 0.5 or lower than -0.5, 28% (9 out of 32 genes) of genes in our study matched the result found in Lauro *et al*. (2009). The other genes did not show a strong correlation with initial nutrient concentration, except for COG3710, which was the only gene that showed the opposite trend from the pattern found by Lauro *et al*. (2009).

*Functional gene categories correlating with incubation time*

Table 3 shows the Subsystem Level 1 and Level 2 genes that have strong (rho>0.5 or rho<-0.5) correlation with incubation time when controlling nutrient concentration. Only the selected Level 2 genes that follow the same (whether positive or negative) pattern with the Level 1 category are shown. Partial correlation results of "Clustering-based subsystem" and "Miscellaneous" were not included. For Level 1 genes, we also drew a heatmap with normalized relative abundance data (Fig. S6).

The regulation and cell signaling gene category was more abundant in the early successional stage. In this gene category, programmed cell death and toxin-antitoxin systems, quorum sensing and biofilm formation related genes were abundant in the early successional stage. The relative abundances of genes associated with cell division and cell cycle decreased over time, but the correlation (rho=-0.407, p=0.001) was weaker than for other genes presented in Table 3. The motility related genes were more abundant in late successional stages.

There were other genes that showed a significant correlations which had not been predicted in our hypotheses. These included genes related to sulfur metabolism, iron acquisition and metabolism, potassium metabolism, metabolism of aromatic compounds, aminoacids and derivatives, and cell wall and capsule for the early successional stage. For the late successional stage, photosynthesis, protein metabolism, respiration, nucleosides and nucleotides, and DNA metabolism were relatively abundant.

*Taxonomic and functional diversity*

In agreement with the findings of Song *et al.* (2016), the Shannon diversity of species assignments of functional genes showed a humpback curve against initial nutrient concentration and incubation time (Fig. 2-3 A and B). However, functional gene diversity does not necessarily show the same pattern found in the case of the diversity of species assignments of functional genes (Fig. 2-3 C and D). In terms of richness, functional gene richness had a positive linear correlation with OTU richness (Song *et al*. 2016) or the richness of species level assignment of functional genes from metagenome (Fig. 4). However in the case of Shannon diversity which indicates species evenness/relative abundances, as opposed

to richness, functional gene diversity had no correlation with OTU diversity nor the diversity of species assignment of functional genes (Fig. S9).

*Taxonomic annotation of functional genome*

We analysed whether the taxonomic association of each functional gene had strong correlations with nutrient concentration or time, to see if they matched to particular genera of bacteria that might be pinpointed as copiotrophic/oligotrophic or r/K strategists. However, no matter what the function, most of the gene sequences derived from the same set of genera that were abundant throughout all the samples (Fig. S7-8), for example, *Acinetobacter, Pseudomonas, Sphingomonas,* etc, such that we were unable to identify any genera as being either clearly copiotrophic vs oligotrophic, or r vs K strategists.

**Discussion**

*Chemical analysis of culture material*

The overall result seems to confirm that as expected, there is some decrease in nutrient availability over successional time, but the initial nutrient concentration is much more important overall than the change over time. The nutrient measured in the culture medium might be derived from the leftover of initial nutrient, or could be derived from recycling of nutrient from dead cells. One important point is that nutrient A had only 2 to 5 times higher DOC compared to nutrient C. Possibly, carbon in nutrient A was removed quickly and sequestered into extra biomass.

*Functional gene categories correlating with initial nutrient concentration (hypothesis A)*

As hypothesized, cell division, RNA metabolism and cell cycle related genes were more abundant in high nutrient concentrations. High abundance of the RNA related gene categories implies the possibility of rapid growth being achieved by the more active expression of overall genetic information encoded in the DNA. This seems to fit with the view that a copiotrophic environment selects for the functions of growth and reproduction at the expense of other gene functions. Conversely, and as we had suggested, it appears that lower nutrient conditions require greater abundances of genes associated with obtaining and sequestering nutrient sources such as carbohydrates. This prioritization of acquisition of nutrients, in different forms and at lower concentrations, suggests that indeed such functions are a fundamental part of the oligotrophic strategy in bacteria. It seems that oligotrophic bacterial species also have relatively high abundance of the genes related to respiration and electron transfer reactions that are important for the utilization of their acquired nutrients.

We also hypothesized that copiotrophs would show a stronger tendency to contain dormancy and sporulation genes, since they are not adapted to actively grow in nutrient poor conditions (Fierer *et al.* 2007). The result supports a view that sporulation is in particular a strategy used by copiotrophs, to shut down the metabolism when nutrient supply decreases, and then wait until nutrient supply improves at some time in the future.

There were also various nutrient concentration-related patterns in other gene categories. For example, virulence, disease and defense related genes turned out to be abundant in oligotrophic conditions. The negative correlation of genes related to resistance to antibiotics and toxic compounds (one of the subcategories of virulence, disease and defense genes) with nutrient concentration supports a view of the greater importance of interference

competition amongst bacterial species at lower concentrations of nutrients (Vance 1984).

Overall, the correlations of COG genes with nutrient gradient in our results – at least those genes that show significant correlations – do largely match the patterns noted by Lauro *et al.* (2009) for copiotrophy vs oligotrophy (Table 2). This is all the more striking when one considers the very different basic design of the two studies, and the fact that the target species and overall community type belonged to two very different environments: Lauro *et al.*'s study (2009) was on marine bacteria, whereas our study was derived from soil. This strengthens the view that many of the genes they pinpointed really are of importance in relation to trophic strategy, and might be universally so – since they show similar trends in such different environments.

*Functional gene categories correlating with incubation time (hypothesis B)*

As expected, cell division and cell cycle related genes were more abundant in the earlier successional stages, although the correlation was weaker than for some other gene categories. This seems to fit with the theory on 'r' strategists (Pianka 1970) that they are selected for rapid growth and reproduction to exploit initial conditions of lower population densities.

However, contrary to our hypotheses, genes related to regulation and cell signaling were more abundant at the earlier time stages of succession in our culture experiments. A priori, the typical view in ecology would be that later (K-strategy dominated) successional stages include more biotic interaction (Morriën *et al.* 2017; Odum 1969), whether by mutualism, interference competition or by specialized parasitism. In our bacterial culture systems, this principle - based upon ecological observation of larger organisms over longer

periods of time - does not seem to hold true. This appears to be an example where principles that apply to macro-organisms do not apply to the microbial world. It is possible, for example, that these genes play a role in a strategy of rapid colonization (in what is largely a two-dimensional static environment across the cellophane surface of our cultures) through biofilm formation. Quorum sensing and biofilm formation related genes were actually more abundant in the early successional stages. In the early successional stages, biofilm formation might involve collaborative interactions between members of the same and different species for the capture of space or nutrients (Shapiro 1998). Also important in initial capture of space or nutrients may be activities preventing other cells from growing, for example, by forming a barrier which can deny access of other cells to the nutrient source, or by antibiotic production (Shapiro 1998).

Within the general regulation and cell signaling category, we likewise found that the subcategory of toxin-antitoxin related genes was more abundant in early stages. These toxin-antitoxin systems in bacterial species are used to provide genetic stability when bacterial species divide (Hu *et al.* 2010). It might be necessary to have high abundance of these genes for faster growing bacteria, since they have very limited time for cell division and may have more errors than slow growing bacteria. Another known function of toxin-antitoxin system as a mean of programmed cell death is to provide DNA through cell lysis as a structural component of biofilm (Bayles 2007; Wen *et al.* 2014). It is possible, then, that in the early successional stage, the toxin-antitoxin system might be used to facilitate biofilm formation genes were likewise more abundant in the early successional stage.

Motility and chemotaxis genes, and especially flagella-related genes, were less abundant in the early successional stages, which also disagreed with one of our hypotheses.

We had predicted that – just as r-selected macro-organisms have features promoting mobility and rapid colonization (Huston and Smith 1987; Peroni 1994) these would also be abundant in the early succession stages of microbial cultures. This might be due to a systematic difference between culture system and the natural environment, whereby pioneering species are required to have high motility. In setting up our culture system, we spread soil elutant all across the culture plates, so early colonizers might not have needed adaptations for dispersal. By contrast, in aquatic systems the flagellum is a key cellular structure for acquisition of nutrients, as there are temporarily nutrient rich patches which bacteria can reach using flagella and chemotaxis (Johansen *et al.* 2002). However, in a culture system such as ours, as colonies deplete nutrients from around themselves, slower growing bacteria might require motility to obtain nutrient from spots that have not yet been colonized.

Many other categories of genes showed a significant trend in relation to incubation time. For example, genes related to minor nutrients such as sulfur, iron, and potassium showed strong negative correlations with incubation time. Iron is often a limiting factor for growth, and such genes might be particularly necessary to supply enough iron for rapid growth (Church *et al.* 2000). K-strategists, in contrast, already have many growth limiting factors other than iron to contend with, so might be subject to relatively weak ecological selection for iron uptake compared to other factors.

Another interesting time-related trend is the strong negative correlation of genes related to metabolism of aromatic compounds with incubation time. Aromatic compounds might be used as a nutrient source providing additional metabolic versatility for r-strategists. Even though aromatics are not likely abundant in our culture media, this may be an incidental legacy of the fact that these are soil bacteria that normally live in aromatic rich (e.g. tannin

and lignin-rich) environments.

*Taxonomic and functional diversity (hypothesis C)*

Over the years there has been much discussion of the hypothesis that species diversity has a positive correlation with stability, due to greater functional diversity (McCann 2000; Wardle *et al.* 1999). However, the observed results in terms of both functional diversity and ecosystem stability vary greatly between different studies, with some studies supporting the hypothesis of a link to taxonomic diversity and others contradicting it (Cadotte *et al.* 2011; Hooper and Vitousek 1997; Mayfield *et al.* 2005; Petchey and Gaston 2006). One of the main reasons for this, is that there is no consensus about how best to define functional diversity – and indeed it is often quite vaguely defined. Functional gene diversity, based on the proportion of reads in different categories, is highly quantitative data, and was defined in this fairly objective way in our study. Gene functional diversity, in terms of functional gene richness and relative abundances showed a clear linear relationship with OTU richness/richness of species within our metagenomes – empirically supporting the supposition that more taxonomic diversity is associated with more functional diversity. We did not test for aspects of ecosystem stability (McCann 2000) or efficiency/rates of ecosystem processes. It would be interesting in any follow-up studies to test the relationship between stability, species diversity and functional diversity in such culture systems. It is also possible that strength of microbial interactions – which might vary in complex ways that do not necessarily follow overall diversity – confounds the expected diversity-stability pattern (Ho *et al.* 2016).

*Taxonomic annotation of functional genome*

Our previous study which used amplicon sequencing gave us possible candidates for taxa previously categorized by us as copiotrophic or oligotrophic, on the basis of their prevalences in different nutrient treatments (Song *et al.* 2016). Beyond this, our metagenome study presented here provides functional genes associated with bacterial strategies in different nutrient concentration and incubation time. The taxonomic annotation of the functional genome shows a similar distribution pattern, particularly in terms of the most abundant genera associated with each functional gene category, (Fig. S7-8) which implies that the difference between samples in terms of functional gene composition is strongly linked to the differences in relative abundance of these most abundant genera. It is also possible that the differences in functional composition between samples might be caused by the combination of many minor genera that has been selected to their functional characteristics. Finer phylogenetic resolution (species or strain level) may be needed to capture differences in the community composition between treatments.

*Possible limitations of this study and suggestions for further work.*

It is important to acknowledge that there are various limitations in this study which may limit the accuracy or relevance of the results in terms of understanding bacterial community ecology in nature:

1) This is an artificial system. Microcosms are by their nature derived and simplified from natural systems. This simplification has the advantage of allowing more precise control and manipulation of conditions, but the risk of leaving out certain crucial aspects of natural

systems which may make their results less relevant to nature. It is possible that microcosms which modify and monitor small quantities of soil (rather than soil elutant) would give a more representative understanding of bacterial strategies in nature. It is also important to realize that the types of nutrients added might have an important effect on community structure. Also, the soil system is only one of a huge variety of environments bacteria can live in. In other environments, different strategies might apply – although the overlap between the genes associated with copiotrophy in our study and those found by Lauro *et al.* (2009) is reassuring, and implies that we may be observing true general patterns in bacterial ecology.

2) We did not study metatranscriptomes. Here we studied the metagenome (DNA), rather than metatranscriptome (RNA) which would provide a more direct picture of cellular activity. It is possible that part of the community composition and functional gene composition that we see is related to a 'legacy' of cells that are already dead or inactive, derived from earlier successional stages or from the initial soil elutant. This may be tending to 'blur' the functional patterns observed. On the other hand, the metagenome can give a picture of the whole range of potentially expressed genes present, which may be relevant to the overall strategies of these organisms in nature, whether or not they were being expressed at the time of sampling.

3) Our data has been collected across a prescribed (though very broad) range of initial nutrient concentrations and incubation times, so the conclusions made in this study may only be applicable within this range. It is possible that the gene abundance patterns would differ outside of this range.

It is likely that the more nutrient-rich treatments we used are much more copiotrophic than the average background level of nutrients in soils, but do correspond to

occasional, spatially isolated and often brief, bursts of very high nutrients from rotting animal carcasses, rotting rootlets, dung, rotting fruits etc. These highly copiotrophic environments are likely to be ecologically important enough to select specialized communities – even if strategists that thrive in these environments must bide their time (perhaps as dormant or low activity forms at lower abundance within the general soil community) between such opportunities. These bursts may also occur on a microscopic scale (e.g. the vicinity of a single dead nematode), but to bacteria they would be high nutrient environments nevertheless.

We did not do chemical analysis for the "nutrient E" sample which has the lowest nutrient concentration. From the dilutions, we can deduce that DOC and DTN in nutrient E should be lower than 3mg/L which is similar to environmental samples (Jones and Willett 2006; Michalzik *et al.* 2001) depending on the environment that is used for comparison.

4) It is difficult to separate the effects of environmental selection and phylogeny. If a particular phylogenetic group of bacteria is particularly abundant in (for example) copiotrophic conditions, any genes that are consistently associated with that group will tend to be more abundant. These genes may be fundamental to the group's success as copiotrophs, or alternatively they might be 'riders' which have no particular significance. Instead, the true 'strategy' might lie with subtle differences in other groups of genes – perhaps involving finely tuned qualitative differences in behavior of expressed proteins rather than differences in gene abundances.

5) It is unclear whether the patterns observed are always due to selection for a particular gene function, or selection against other functions within the genome. Since metagenome composition measures relative abundances, a gene may become more common not because it is actively selected for, but by the relative absence of other sets of genes which are selected

against. This somewhat limits the confidence that can be placed in the conclusions.

6) Since the percentage of proteins with no known function was greater in later successional stages, it is possible that there is a trend of increasing diversity in gene functions and functional composition with successional time that cannot easily be discerned using existing databases.

Nevertheless, despite all these caveats we regard this study as a useful early step towards identifying the key differences which can explain the differences in bacterial community composition along nutrient and time gradients.

**Conclusions**

Although based on a highly simplified system, the results of this experiment identify sets of genes which may be part of distinguishable community-level strategies seen amongst bacteria, in terms of the relative abundance of particular functional gene types. In terms of the oligotrophic-copiotrophic axis, the gene abundance patterns we found are close to those identified by Lauro *et al.* (2009), and the agreement between the two studies (based on quite different approaches) reinforces confidence that the functional gene differences found have relevance in determining strategies. We were also able to identify a second axis of functional gene variation, distinct from the oligotrophic-copiotrophic one, that relates to time and resembles the r-K axis found in successional ecological systems of larger organisms. However, it is interesting that the patterns of adaptations (e.g. for dispersal, or regulation and cell signaling) do always not match those classically described for macro-organisms. These results, while intriguing, are merely an initial attempt at discerning and understanding the

basis of bacterial adaptations for survival. It is hoped that this study will provide a spur for thought and further work on this important aspect of microbial ecology.

**Funding**

This work was supported by the National Research Foundation of Korea (NRF) grant [No. NRF-0409-20160032] funded by the Korea government (MSIP).

**Conflict of Interest**

The authors declare no conflict of interest.

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