Microbial Ecology

Microbial Astronauts: Assembling Microbial Communities for Advanced Life Support Systems

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Abstract

Extension of human habitation into space requires that humans carry with them many of the microorganisms with which they coexist on Earth. The ubiquity of microorganisms in close association with all living things and biogeochemical processes on Earth predicates that they must also play a critical role in maintaining the viability of human life in space. Even though bacterial populations exist as locally adapted ecotypes, the abundance of individuals in microbial species is so large that dispersal is unlikely to be limited by geographical barriers on Earth (i.e., for most environments ''everything is everywhere'' given enough time). This will not be true for microbial communities in space where local species richness will be relatively low because of sterilization protocols prior to launch and physical barriers between Earth and spacecraft after launch. Although community diversity will be sufficient to sustain ecosystem function at the onset, richness and evenness may decline over time such that biological systems either lose functional potential (e.g., bioreactors may fail to reduce BOD or nitrogen load) or become susceptible to invasion by human-associated microorganisms (pathogens) over time. Research at the John F. Kennedy Space Center has evaluated fundamental properties of microbial diversity and community assembly in prototype bioregenerative systems for NASA Advanced Life Support. Successional trends related to increased niche specialization, including an apparent increase in the proportion of nonculturable types of organisms, have been consistently observed. In addition, the stability of the microbial communities, as defined by their resistance to invasion by human-associated microorganisms, has been correlated to their diversity. Overall, these results reflect the significant challenges ahead for the assembly of stable, functional communities using gnotobiotic approaches, and the need to better define the basic biological principles that define ecosystem processes in the space environment.

Microbes in Space Are Not Just Accidental Tourists

As we seek life's signature beyond Earth, one essential directive for the missions is to prevent forward contamination of our destination by terrestrial microorganisms [82, 97]. A strict containment requirement, however, conflicts with our desire to establish a human presence beyond Earth. If humans are to travel in space, then microorganisms will be transported by, and coexist with, them—no alternatives exist. In addition to their ''accidental'' introduction as commensal tourists attached to human and spacecraft surfaces, microbial communities will be purposively introduced if bioregenerative systems employing plants and/or biological waste processing are employed for life support (Table 1). The ubiquity of microorganisms on Earth in close association with all other organisms predicates that they must also play a critical role in helping to maintain the viability of the same macro-organisms in space.

In recognition of the need to enable human survival during extended space missions, the National Aeronautics and Space Administration (NASA) supports research and technology development in bioregenerative and physical–chemical life support systems through the Advanced Life Support (ALS) element of the Advanced Human Support Technology program. One of the challenges to developing life support systems is the ability to engineer a homeostatic system in which optimal levels of performance can be maintained over long periods. One

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Table 1. Potential microbial reservoirs in the spacecraft ecosystem

Microbial reservoir	*Unit bacterial density*[a] *(CFU per unit mass, area or vol)*	*Bacterial density*[b] *(CFU per crew member)*	*Bacterial diversity*[c] *(no. species or divisions)*	*Ref*[d]
Human			>10 divisions	
Gastrointestinal tract	10^{11} mL^{-1}	10^{14}	>400 species	(1)
Skin	10^{3}–10^{4} cm^{-2}	10^{8}	>50 species	(2)
Plant			>20 divisions	
Rhizosphere	10^{6}–10^{12} g^{-1}	10^{9}–10^{15}	>4000 species	(3)
Phyllosphere	10^{5}–10^{8} g^{-1}	10^{7}–10^{10}	>85 species	(4)
Bioreactor	10^{7}–10^{11} mL^{-1}	10^{12}–10^{15}	≥13 divisions	(5)

[a]Unit bacterial density estimated from published data for each respective environment on Earth. Where available, the minimum and maximum for each density distribution are provided.

[b]Total microbial density per each individual human crew member assuming 1 kg of plant biomass and 10 L bioreactor liquid volume per person.

[c]Microbial diversity determined by richness measures obtained from either cultivation-based enrichment methods or cultivation-independent molecular methods.

[d](1) Estimates of commensal and transient bacteria in the human gastrointestinal (GI) tract are based on references cited by Whitman et al. [102], Berg [5], and Savage [83]. Among the human-associated bacteria, >99% are found in the GI tract and >75% of sequences in clonal libraries from the human GI tract do not correspond to known organisms in the SSU rRNA database [38, 88].
(2) Estimates of human skin surface-associated bacteria are derived from the cultivation-based data reported by Whitman et al. [102] and the culture-independent diversity data reported by Frank et al. [28].
(3) Rhizosphere bacterial densities have been reported to be 10^{8}–10^{12} cells g^{-1} of root dry mass [87, 95]. Estimates of bacterial diversity in the soil are based on the culture-independent DNA hybridization data of Torsvik et al. [93] and cultivation-based estimates reported by Whitman et al. [102], using the data of Paul and Clark [71] and Gray and Williams [33].
(4) Phyllosphere bacterial densities have been reported to range from 10^{5} –10^{8} CFU g^{-1} of leaf material [50, 61] or 10^{4} –10^{6} CFU cm^{-2} of leaf area [40]. The estimates reported here are from the culture-based estimates of Whitman et al. [102] reported by Corpe and Rheem [18]. Phyllosphere diversity has been estimated to range from >78 to 85 bacterial spp. representing 37 bacterial genera by Lindow and Leveau [53] and by Yang et al. [104] based on the culture-based estimates of Legard et al. [52] and Thompson et al. [90].
(5) The estimate of bioreactor bacterial density is based on the culture-based microbial load of activated sewage sludge in wastewater treatment plants [7]. Estimates of bioreactor diversity are based on the data reported in Wagner et al. [99, 100].

aspect of the homeostasis of bioregenerative systems is the physiological control of a limited number of plant species used for food production. Homeostasis of the microbial components, including the plant-related commensals as well as bioreactors for recycling liquids and solids, will involve both physiological and ecological processes associated with communities comprised of billions of individuals and thousands of species (Table 1). Evolutionary dynamics are an additional consideration given the potential for rapid change in populations of microorganisms through mutation and natural selection (processes that may be accelerated under space conditions). Deleterious functional or compositional changes in the microbial communities (due to physiological, ecological, or evolutionary factors) could lead to reduced yield in the food production systems, the failure of waste recycling systems, the outbreak of latent or emerging disease among the crew, or changes in efficiency of the digestive systems of the crew thereby making the nutrition carried in food supplies unavailable to consumers.

The complexity of factors associated with homeostatic control of microbial systems, and the concerns with potential deleterious imbalances, suggests that the engineering, or assembly, of microbial communities is an important but largely unexplored consideration in long-term human spaceflight. The following is a review of what we believe to be the relevant underlying issues, including recommendations on the necessary experimental studies to improve our understanding.

Microbial Evolution in Space

Microorganisms, because of their small size, vast numbers, rapid growth rates, and comparatively simple genetic systems, tend to be modified rapidly under conditions that are favorable for mutation and natural selection. On Earth, the overwhelming diversity of microbial types and their ability to survive in extreme physical environments (i.e., extreme to humans, at least) suggests that few niches have remained unoccupied during the many millennia of microbial existence. As a result, newly evolved organisms are often unable to compete for niche space with established populations even when imbued with phenotypic differences that would confer a selective advantage in the colonized niche. Despite the prokaryotic propensity for being everywhere, spacecraft are not arks and it is unlikely (indeed, the existence of pathogens suggests that it is undesirable) that all types of microorganisms from Earth will be carried along into space to colonize new habitats. There will exist large numbers of unfilled niches and unexploited resources available for extant or newly evolved microbes in the isolated ecosystem of the spacecraft. Bacteria are poised to divide into new species. With great frequency, a bacterial population can divide into ecologically distinct populations, ecotypes, which can then diverge without bound over evolutionary time [12–16]. In the bacterial world, evolution of new species is limited only by the rate at which a population generates new genotypes that are

ecologically distinct. The rate of molecular evolution and generation of new genotypes is ultimately determined by the rate of mutation. Speciation in bacteria, unlike the highly sexual plants or animals, is not hindered by genetic exchange between incipient species. Therefore speciation should be expected to be much more frequent in bacteria than in plants and animals, and indeed, microcosm experiments have shown speciation to occur within weeks [94] or even days [75] in relatively simple environments.

In the environment of space, rates of both gene transfer and mutation may be elevated, due in part to lowered mismatch repair activity [41, 105]. Higher mutation rates could contribute to a higher rate of speciation, since each ecologically distinct mutant can potentially found a new species. Also, reduced mismatch repair could change the dynamics of recombination [98] so that DNA from more distantly related species is more easily recombined into a given recipient. An elevated inter species recombination rate may foster the evolution of new species in two ways. First, uptake of nonhomologous genes from distantly related species is thought to be a major mechanism by which a bacterial lineage acquires new ecological properties [61]. In some instances, newly acquired properties may include the ability to become pathogenic or to express increased virulence by the acquisition of a new function. Second, recombination between newly divergent species can pass adaptive mutations between them and can promote the populations' extended coexistence or persistence within an environment [15]. This would enable the maintenance of genetic diversity within and among populations even in the face of repeated diversity-purging episodes of periodic selection.

The Fundamental Microbial Niche in Space. Since the early part of the 20th century, ecologists have recognized the importance of the niche in structuring communities of organisms at all levels. However, even the first advocates of niche theory defined the concept differently among themselves. Whereas Grinnell [35] defined the niche as the sum of the environmental variables existing at a point in space and time, Elton [25] considered the niche as the functional role of a species in the community. An important consequence of Grinnell's concept is that it is possible to have unoccupied niches. Later, Hutchinson [44] combined aspects of both Grinnell and Elton by defining the niche as the *n*-dimensional environmental (hyper-)space that is or can be occupied by a population (see Griesemer [34] for a review). Hutchinson's definition is similar to Grinnell's in that it allows for niches to exist independently of the presence of any resident organism. Thus, Hutchinson speaks of *fundamental niches*, those that exist with or without occupation by a locally present target species, and *realized niches*, those that are, in fact, occupied. While most ecologists accept the niche concept in general, there remains controversy surrounding the definitions, particularly involving the existence of unfilled niches [34] and the inclusion of facilitation (i.e., positive interactions between organisms in the niche) as a mechanism affecting niche structure [7]. If the niche concept is valid (in any of its forms) it should operate for microbes as well as for any other category of life. The small size of microbes makes demonstration of the concept on a scale that is relevant to ecosystems nearly impossible. Individual microbial cells interact with one another only over short distances, perhaps a few hundredths of a millimeter, controlled by diffusional processes. Thus, direct competition likely occurs only at those relevant scales. At greater distances, a different species may be so similar to the focal species in the particular habitat that the two would compete directly if they were in close proximity. The lack of competition at ecologically relevant scales can explain a number of phenomena observed in natural microbial ecosystems. In particular, it contributes to the hyperdiversity that microbial communities display in many terrestrial ecosystems. Even using the lower-limit diversity estimates of Hughes et al. [43] for grazed grassland soils, species richness is on the order of 500 "species" or OTUs per gram of soil. Using upper-limit estimates of "species" diversity calculated by Curtis et al. [19], it is inconceivable that that there could be nearly 40,000 realized niches in each gram of soil. There must be other explanations for the hyperdiversity of microbial communities, and scale of the organism is likely central to the issue.

Given the small size of the organisms and the tendency to attach to surfaces whenever the opportunity arises, habitats such as surface soil or plant root surfaces, reactor walls, etc, should be able to support a large number of different species that would seem to have significant niche overlap (at least for a significant subset of their total functional ability). Indeed, microbial ecologists often use the ambiguous term "microhabitat" to describe the scale-dependent spatial insulation of competing species. For example, bacterial development in soils may be influenced by conditions within only a few micrometers, whereas a hyphal fungus can extend its immediate surroundings in much the same way that a plant root system does. For this reason, a fungus may experience a degree of averaging of soil conditions and is not restricted to as small of a microhabitat as a bacterium [70]. In aquatic systems, the more diffuse nature of the environmental matrix and increased resource mixing may mean that microbes are affected by environmental variability existing at a broader spatial scale, compared to a more highly structured soil matrix. The size of a microhabitat may be defined by the physical and chemical environment directly adjacent to the microbial cell or colony [70] and, in this regard, is not a fixed quantity; its size is operationally dependent upon the specific process

or microorganism under study, and the nature of the environmental matrix within which the organism resides. In soil for example, direct competition among microbial types is limited by spatial isolation and resource heterogeneity among habitats resulting in natural microbial communities where individual bacterial species are equally abundant and uniformly distributed [21, 106]. It is unclear whether this distribution is prevalent in other microbial ecosystems.

Given the very patchy nature of microbial distribution and resource availability, the primary control on the success of an invading species may be the probability of falling within the interaction distance of a competitor. Fundamental niches are spatially and temporally distributed, i.e., gaps between realized niches, such that space and resources are often available for invaders if they land in the right spot at the right time. It is often noted that the potential success of an invading species, or infectivity in a clinical setting, is related to inoculum size [2, 6, 9, 10, 54, 78, 96, 101]. This might simply be explained as an increased probability of some individuals landing in a location that is either uninhabited or underexploited by a competing species. Similarly, one could argue that a highly diverse community with substantial, spatially discrete niche overlap can minimize the probability of an invader avoiding a competitor, because a highly diverse community would tend to maximize the ubiquity of competitive characters within the macrohabitat.

Microbial Succession in Closed Systems. Temporal changes in community composition resulting from species replacement (i.e., succession) will occur in spacecraft just as they do in natural systems. Successional dynamics are governed by both the availability of species (introduction of new types by either mutation or recruitment) and their performance within the system (selection). Mechanistic models for the differential performance of species over time within communities include gradient-in-time [72, 103] and competitive sorting [58, 59]. The former is based on the concept, that species are differentially adapted to the variation in physiochemical factors along a temporal gradient and is more appropriate in systems where the environment is modified over time, potentially by the action of early colonizers (i.e., process of facilitation). The latter model predicts that initial community composition is based largely on stochastic colonization events, but that poor competitors are "sorted out" with time. Both models predict that over time, community composition will become more predictable and that the average niche breadth of individuals will decrease resulting in an increasing proportion of specialists. One measure of community response, CLPP or community-level physiological profiling [63], has been applied for the measurement of community niche breadth within ALS plant production systems. Previous tests with prototype bioregenerative systems have supported the predictions regarding decreased niche breadth over time, based on the rate of extinction of community phenotypic characteristics [30] and the proportion of culturable types [31] as indicators of niche specialization. The decline in the number of culturable cells does not appear correlated to the physiological shift of culturable cells to a nonculturable state, but rather changes in community composition to cell types with more specialized growth requirements that prevent their rapid growth into colony-forming units (CFUs) on typical solid agar media. Tests with prototype plant growth systems using recirculating nutrient film technique hydroponics (NFT hydroponics) further supported the competitive-sorting model of succession. The rhizosphere communities associated with potatoes (*Solanum tuberosum* L.) fed from separate nutrient delivery systems were highly variable at the onset of the studies, reflecting stochastic colonization events, but became increasingly similar (i.e., more predictable) over time as determined by CLPP (Fig. 1A). An even more dramatic temporal shift in community composition was evident in the rhizosphere of hydroponically cultivated dwarf wheat (*Triticum aestivum* L. cv. USU-Apogee), shown in Fig. 1B. The temporal shift in the rhizosphere communities of both potato and wheat was accelerated on the roots of young plants placed into the recirculating nutrient delivery systems containing older plants, indicating that "competitively" sorted microbial species from the older roots colonized the young roots. Facilitation of the rhizosphere by early microbial colonists was not necessary. These data suggest that the use of "competitive sorted" communities from established ground analogs may be used as inocula for spaceflight systems to increase the speed and predictability (and perhaps reliability) of successional dynamics. However, the high proportion of nonculturable types within the established communities (data not shown) indicates that the use of known mixtures of microbial isolates (i.e., gnotiobiotic approaches) as an inoculum source would be of limited value due to the present inability to culture important community members. Culturability, measured as the ratio of cells detected by CFU formation on R2A agar (Difco, Detroit, MI) to those detected by acridine orange direct count, declined from 30% at planting to only 3% by day 63 in the wheat rhizosphere communities (data not shown). Of the 52 or so divisions that comprise the domain Bacteria, only 26 are currently represented in pure cultures [20, 77]. Even though new methods hold promise for the isolation of some of the "uncultured" fraction in some environments [46], it is not clear if even a partial solution is at hand for most environments. This points to several serious limitations of gnotobiotic approaches for ALS bioregenerative systems that have been previously

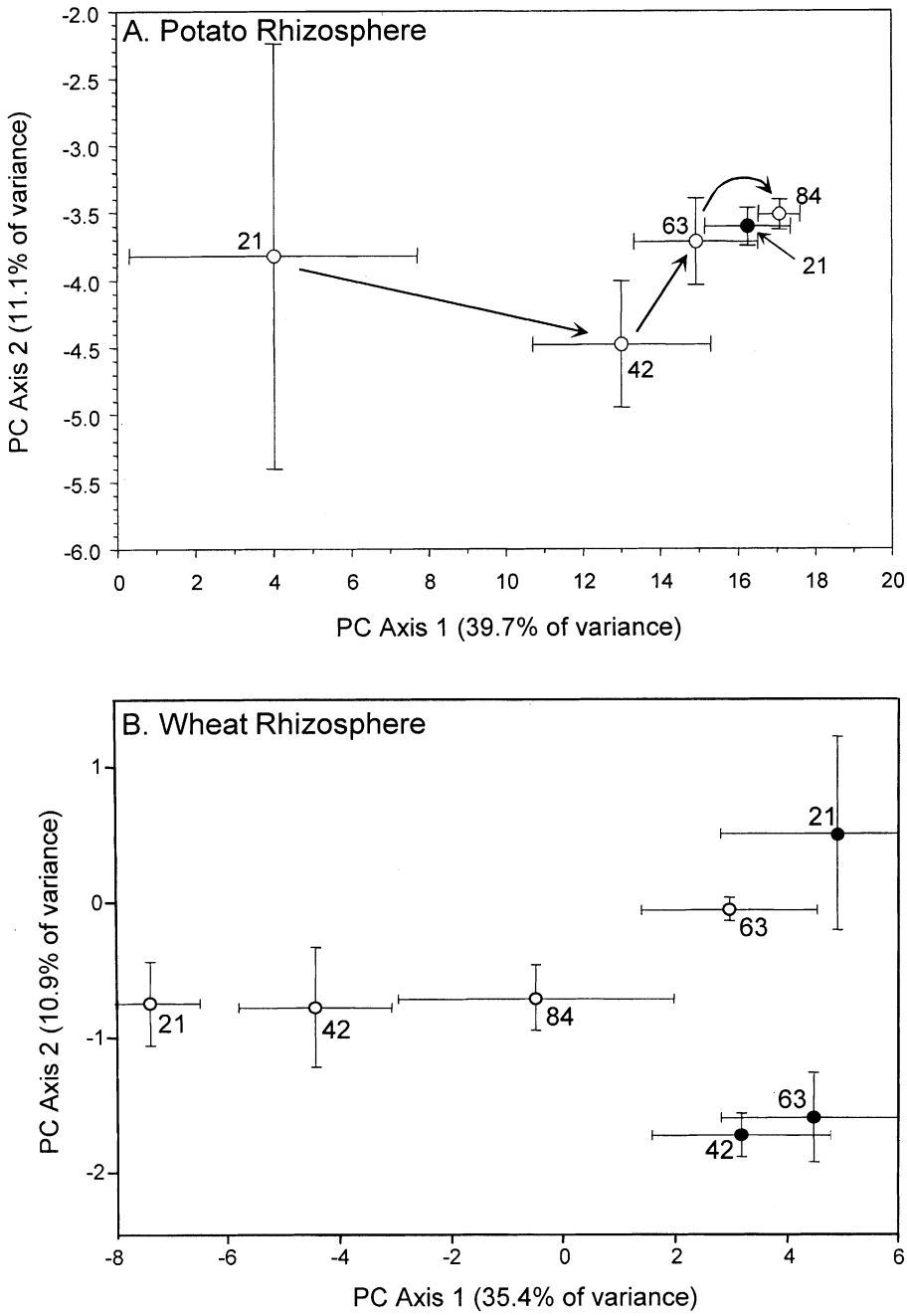

Figure 1. Principal component analysis of community level physiological profiles (CLPP) in the rhizosphere of two hydroponically grown crops: (A) potato and (B) dwarf wheat. The number adjacent to each data point indicates the day of rhizosphere sample collection after planting. Open circles represent rhizosphere samples collected from the intitial planting; closed circles represent rhizosphere samples collected from the sequential planting. Temporal shifts in bacterial community composition are indicated by arrows.

addressed by Garland et al. [31]. Despite these limitations, the relative merits of the gnotobiotic approach for ALS systems make worthy their continued exploration.

On the Earth, the large diversity and wide dispersal of microorganisms suggests that if a niche in a given ecosystem is unfilled, microbes from surrounding habitats that are capable of filling that niche will quickly do so. In closed or otherwise insulated systems, including spacecraft or planetary surfaces, no "infinite species pool" exists from which to draw competent organisms to occupy unfilled niches. In such a case, successional dynamics will be more dependent on community change caused by evolution of populations—mutation of existing organisms and selection of those traits in the new genomes that able the mutated organisms fit to fill the empty niche. This might lead to the hypothesis that succession within closed systems will become a much slower process since it depends on mutation rather than recruitment as the primary driving force.

Experimental evidence suggests that generalists are favored when extreme conditions (e.g., low pH, low nutrient concentrations, heavy metals) limit the number of tolerant organisms [1, 62]. It is unclear from present knowledge if limiting recruitment via insulation to in-

vasion, rather than limiting physiochemical conditions, would also favor generalists, although one would predict that organisms with greater metabolic versatility should be able to utilize more of the available resources in these systems. Chemostat experiments with only a single bacterial species (an extreme case of insulation with respect to invasion) found niche partitioning even in a single dimensional niche (i.e., glucose as the sole carbon source) [21, 75, 81]. After 800 generations, three distinct specialist strains of the original *Escherichia coli* progenitor had developed which were adapted to glucose or either of two compounds leaked by *E. coli* during glucose oxidation, acetate and glycerol [81]. These results, along with those of Dhykuizen and Dean [21], suggest that the evolution of microbial communities may tend toward increased specialization and niche partitioning even when recruitment is severely limited. Rainey and Travisano [75] reported a similar result for *Pseudomonas fluorescens* when incubated in static broth cultures versus shaking flask cultures. Spatial structure was demonstrated to be the key factor responsible for the emergence of polymorphic populations in the static cultures while the homogeneous shake flask populations remained genetically uniform. Spatial isolation has also been implicated as the primary factor determining microbial community diversity in soil [106] although resource heterogeneity and negative frequency-dependent selection cannot be eliminated as important contributing factors [15, 17]. Examination of communities over gradients of recruitment and niche complexity (e.g. spatial structure and/or environmental fluctuation) is required to better understand the processes involved [43]. What is unequivocally clear, however, is that bacteria are poised to divide into new species. Given that the demarcation of microbial species boundaries may be the simple genetic ability to occupy different niches [23] it is not surprising that microbial diversity is so high.

The balance between specialists and generalists may have important ramifications for overall system stability since stochastic extinction in a community dominated by generalists may limit community function to a greater degree than in a community comprised of specialists. The severity of this outcome, however, depends upon two ecosystem components: (1) the degree of degeneracy (functional redundancy) within the system, and (2) accessible ecological opportunity (afforded by spatial structure) within the community [26, 27, 37, 65, 75, 80]. The trajectory of ecological succession will be constrained by selective pressures exerted by the physical, chemical, and biological (other organisms present) conditions that exist within the niche. Organisms must utilize resources available within a habitat, or they must alter those resources to make them available to members of the community. On Earth, adequate evolutionary time has passed to allow a large variety of reactions to develop in individual populations. Reactions necessary for community operation in a given ecosystem can be added simply by emigration of one or more organisms from the massive species pool that exists. Note that the addition may not be instantaneous, but the large number of organisms able to carry out individual biochemical reactions makes the probability of finding a suitable organism in a reasonable time very high—Beijerinck's rule, ''Everything is everywhere, the milieu selects,'' [3] seems to hold true on Earth. Space conditions limit the pool of species that can be included in the ecological system to effect all its functions to those included at the time of launch (in the case of enclosed terrestrial habitats) plus any that form as a result of mutation once the vehicle has been sealed. In order to gain insight into the development of complexity and stability of degenerate biological systems in the space environment, the experimental system must enable long-term microbial cultivation in a controlled, reproducible environment that enables tracking the evolution of populations and communities through time.

Neutral Models of Molecular Evolution and Community Biodiversity

Evolution requires mechanisms that generate new genotypes (mutation and recombination) as well as mechanisms to eliminate existing ones (selection and genetic drift). Natural selection is powerless to discriminate between variants unless the genetic variation alters traits that affect reproductive success. Consequently, the rate of adaptive evolution may be limited not by selection but instead by the appearance of new genotypes. Since most mutations are either neutral (no fitness effect) or deleterious (negative fitness effect), the mutation rate is under constant pressure to decrease by natural selection. The rate of mutation is determined by a trade-off between natural selection favoring lower mutation rates to maintain favorable combinations of alleles and opposing selective forces favoring higher mutation rates to generate novel allelic combinations. In an isolated environment such as a spacecraft, populations with low mutation rates might perish for lack of sufficient adaptive variation while those with higher mutation rates would suffer from an excessive load of deleterious mutations [48]. Selection against mutators—microbes with an enhanced rate of mutation (i.e., hypermutable phenotype) and an increased tolerance for recombination with divergent sequences (i.e., promiscuous phenotype)—limits their frequency in most natural populations to <0.01% of the total population, except where selection favors novel phenotypes, e.g., pathogenic microorganisms.

The neutral theory of molecular evolution holds that most nucleotide substitutions in DNA do not result in amino acid replacements, and those base mutations that do generate amino acid substitution have little or no

impact on the fitness of the organism [49]. By integration of Kimura's theory with the equilibrium theory of island biogeography of MacArthur and Wilson [56, 57], Hubbell has provided a framework for the modeling of community diversity at an ecological scale [42]. Essentially, the number of established species within a community (richness) is determined by a dynamic balance between the rate of speciation and the rate of extinction of community members rather than by the number of fundamental niches that are available for exploitation. If the tenets of the theory are correct, then experimental designs are possible that can determine the rules of community assembly in relatively simple ecosystems such as batch culture shake flasks, ALS bioreactors, or perhaps even wastewater treatment plants.

Degeneracy of Biological Communities

In the context of biological systems, degeneracy is defined as "the ability of elements that are structurally different to perform the same function or yield the same output" [24]. This differs from redundancy in engineered systems in that functions are not duplicated by identical elements designed to limit the impact of system component failures. The distinction is not merely one of semantics as it amplifies an even more profound distinction between natural and engineered systems. Where engineered systems are intelligently designed to accomplish a task with the absolute minimum of interacting parts, biological systems are neither intelligently designed nor limited in the complexity of their interactions. As products of natural selection, degenerate elements (whether species, genes or enzymes) engender biological systems with a near-limitless potential for phenotypic diversity. Complex and unpredictable phenotypic changes, both adaptive and nonadaptive, arise during the diversification of bacterial types because of stochastic interactions that manifest complexity across multiple levels of organization. Degenerate biological systems are invariably complex systems. Because the generation of variation is a random process, identical microbial types in identical physical environments may independently develop unique solutions (adaptations) to the same problem. Each independent solution may derive from a unique phenotypic change yet confer the same relative competitive advantage (fitness) in the environment. In the vocabulary of population genetics, multiple equally probable adaptive peaks may exist for each evolutionary pathway. Unlike redundant systems, each random change within a degenerate system has the potential to elicit an independent, unpredictable response to environmental change. Just as each of us is the unique consequence of the interaction between genes and environment, biological systems are composed of individual elements (species) whose ultimate success (fitness) is determined by the interplay between the products of their genome and the physical parameters of the environment (niche) in which their genes are expressed.

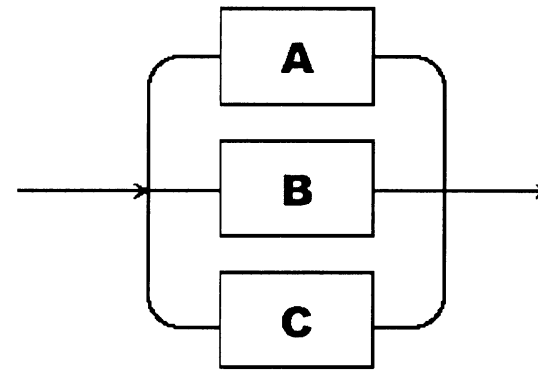

Figure 2. Congeneric homotaxis as described by Hill and Weigert [39]. Figure from Odum [68].

Microbial communities on Earth are inherently degenerate. In this case, as shown in Fig. 2, stability (or resilience) is conferred on an ecosystem because multiple microbial types are capable of carrying out a given function in the ecosystem, presumably across a wide range of environmental conditions. If one of the organisms is eliminated from the ecosystem, or if the organism ceases to function for any reason, another organism present within the system (hence, congeneric, across genera) may occupy the open niche and function within the system allowing maintenance of functional ability at or near the level prior to the loss of the first organism (i.e., homotaxis [39]). Although the fidelity of the aggregate community process is replicated by the function of the congeneric organism, there are no biological constraints requiring that the output be exactly duplicated by the replacement. Community complexity may increase over time as degenerate elements may generate outputs (by creating new combinations of organisms within communities) that differ from those of the original community function [24]. The converse, however, is also possible and the community may hemorrhage both biological diversity and functional complexity by enabling the invasion of an organism that was competitively excluded by the first organism.

The vast potential for degeneracy and hyperdiversity among microbial communities (described in Fig. 3) on Earth can be easily estimated if we consider the approximate number of genomes in, say, a gram of soil. Torsvik et al. [93] used DNA reannealing kinetics to show that the total number of *Escherichia coli*–sized genomes in a 1-g sample of an agricultural soil was ~4000–7000 depending on the effort taken to purify the extracted nucleic acids. The size of an average *E. coli* genome [66] is 4720 kbp. The average molecular weight of an *E. coli* protein (40 Da) divided by the average weight of an amino acid (110 Da) in *E. coli* divided by 3 (the number

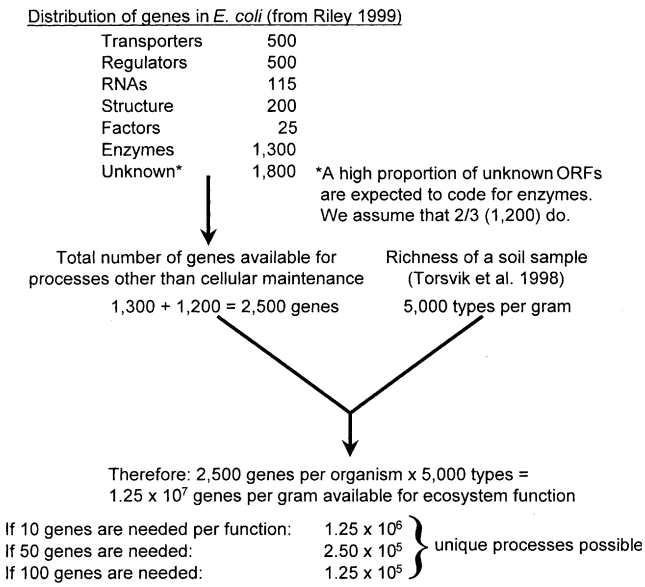

Figure 3. Calculation of theoretical values of degeneracy in bacterial communities.

of nucleotide bases needed to code for each amino acid in a protein) sets the maximum number of different proteins that can be produced by the *E. coli* genome at 4300 [66]. However, because some of the DNA is needed to code ribosomes and t-RNA and to provide intragenic control sequences for protein synthesis, Neidhart et al. [66] estimate a realistic upper level for the number of possible proteins in *E. coli* to be 3800. If we assume that half the enzymes formed are used for "non-ecosystem" functions such as cell synthesis and organization, 1900 enzymes are left for ecosystem-type functions such as nitrogen mineralization and carbon oxidation. Riley [79] did a similar calculation with *E. coli* and determined that there are 1300 enzymes carrying out metabolic reactions. Furthermore, she determined that there are 1800 open reading frames (ORFs) for which the purpose is unknown. She assumed that a high proportion of those ORFs should be expected to code for enzymes. If we assume that two-thirds of the ORF code for enzymes, then the total number of enzymes available for ecosystem functions is ~2500, a value which corresponds well with the calculation based on Neidhardt's [66] assumptions. Continuing with the Riley data, multiplying the number of enzymes potentially available for ecosystem function in a single genome times the number of genomes in the gram of soil, assumed to be 5000 here, based on Torsvik et al. [93], yields a total of 1.25×10^7 genes. Depending on the number of enzymes required to carry out a single function, there are between 1.25×10^5 and 1.25×10^6 potentially unique functions that could be carried out by the community in the gram of soil. It seems inconceivable that such a large number of functions exist in the small volume that is 1 g. Clearly, there must be a large number of organisms capable of carrying out each function. Microbial communities must therefore be degenerate and complex systems.

Degeneracy in microbial communities is commonly observed whenever plating of bacteria is done using selective media containing even a single carbon/energy source. Rarely is only a single isolate obtained (especially when using common substrates such as starch, cellulose, or a specific protein or lipid). If simple communities develop in the closed systems proposed for use in Advanced Life Support (ALS) systems and the inevitable invasion of planetary surfaces upon human contact, the systems might be inherently unstable. They are likely to fail (in terms of system functions) and are likely to be prone to invasion by organisms from other systems or by new organisms generated as a result of mutation.

Why Complexity and Degeneracy Matter

Macroecological theory predicts, and empirical data have shown, that community stability (i.e. resistance to physical disturbance or invasion and the concomitant loss of ecosystem function) is generally higher at intermediate levels of species diversity [91, 92]. Although the interpretation of this result and its causes is equivocal and not without controversy (see the review by Loreau et al. [55]), there is consensus among ecologists that a large pool of species is required to maintain ecosystem function. However, does this relationship hold true for microbial communities whose composition may be exceedingly diverse in phylogenetic space (i.e., species richness is very high) yet are degenerate with respect to function?

Ecosystem function is a concept that may be defined as any relative or absolute measure of productivity within the system, e.g., CO_2 flux, biomass flux (accumulation or decomposition), or percent substrate utilization. Biodiversity is a concept generally defined by measures of species richness (the number of different species) and evenness (the relative abundance of each species) although other measures have been applied, i.e., functional diversity. The relationship between ecosystem function and biodiversity has not been well defined for any ecosystem, microbial or otherwise. There may in fact be no relationship at all, the null hypothesis, whereby ecosystem function is not related to the number of species in an ecosystem. If a relationship between function and richness is observed, the observed pattern may define the nature of the relationship. If varying the number of species in the ecosystem has no effect upon function (the null hypothesis), no relationship exists between biodiversity and ecosystem function. If, however, a positive correlation is observed between biodiversity and function, the shape of the curve enables discrimination between alternative hypotheses describing the process responsible for the observed pattern. A linear curve

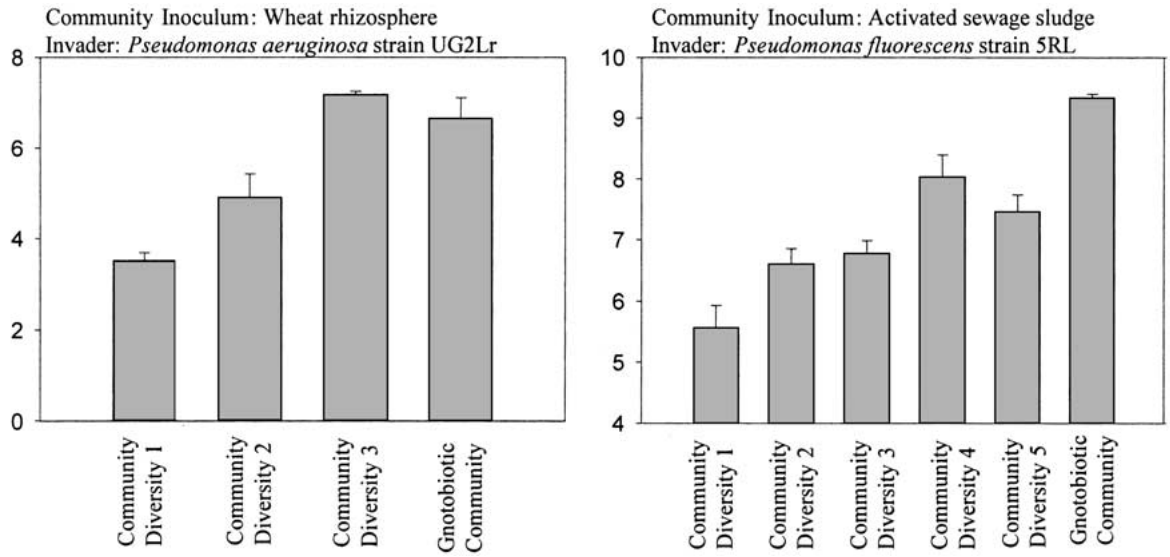

Figure 4. Relative density at harvest of *Pseudomonas* spp. introduced into the rhizosphere of dwarf wheat (*Triticum aestivum* L. cv. USU-Apogee) in hydroponic microcosms. A single strain of either *Pseudomonas aeruginosa* UG2Lr or *Pseudomonas fluorescens* 5RL was inoculated into the simulated rhizosphere of a 1 L microcosm containing dwarf wheat. The initial population density of introduced *Pseudomonas* spp. was $\sim 1 \times 10^8$ CFU mL^{-1}. A community richness gradient was created in the rhizosphere of each diversity treatment by serial dilution of a microbial community inoculum obtained from either (A) wheat rhizosphere or (B) activated sewage sludge. Each community diversity level reflects a 10-fold dilution of inoculum. The rhizosphere gnotobiotic community was assembled from a hydroponically cultivated wheat rhizosphere in biomass production studies at Kennedy Space Center, FL. The wheat rhizosphere isolate library was screened on R2A agar plates (Difco, Detroit, MI). The activated sewage sludge gnotobiotic community was assembled from a collection of strains identified in a clonal library of SSU rRNA sequences obtained from a waste water treatment plant [17].

supports the rivet hypothesis describing a direct correlation between diversity and function. Any decrease in biodiversity results in a proportional decrease in ecosystem function. A more curvilinear response supports the redundant species hypothesis and reflects an ecosystem where the loss of diversity does not affect ecosystem function until biodiversity reaches a threshold. If only a few key species remain and one of these species is lost, ecosystem function may rapidly decline or fail entirely. The idiosyncratic hypothesis reflects a relationship where ecosystem function changes when species richness declines, but the response is unpredictable. Idiosyncratic variation in ecosystem function in response to changes in richness would be indicative of limitations in our knowledge about the diversity of the system. Either the efforts to sample biological diversity in the system are inadequate (i.e., we cannot capture or analyze extant diversity because it is too high) or our understanding of the interactions between species or guilds within the system is flawed (i.e., we cannot describe the rules of community assembly in the system).

Negative frequency-dependent selection is one attribute of experimental bacterial populations in nonequilibrium conditions that results in a selective advantage for genotypes when rare but not when they are common. This selection results in the evolution and maintenance of stable polymorphisms within even simple communities that enable the stable coexistence of genotypes that may exceed the finite number of limiting resources [76]. Microbial communities, in effect, evade competitive exclusion and harbor multiple redundant types within "simple" environments, even those initially composed of a single substrate. Although ecosystem stability within microbial communities is dependent upon population composition, the redundancy of functional types within the community serves to dampen the loss of ecosystem function despite frequent oscillations in population density and composition resulting from interspecific competition and genetic drift.

Empirical tests of the importance of diversity on the stability of microbial communities are complicated by the present difficulty in culturing the majority of bacteria found in nature. Communities assembled by combining different numbers of known isolates (i.e., gnotobiotic communities) may be poor analogs of diverse natural assemblages composed of culturable and nonculturable types. We have attempted to avoid this problem by creating gradients in community diversity through dilution/extinction [29, 31, 45]. This approach is based on the concept that diluting an environmental sample, thereby causing rare members to become extinct, decreases the richness of species in the original community. The series of dilutions are introduced into a system of interest, and the resulting communities are treated as different levels of

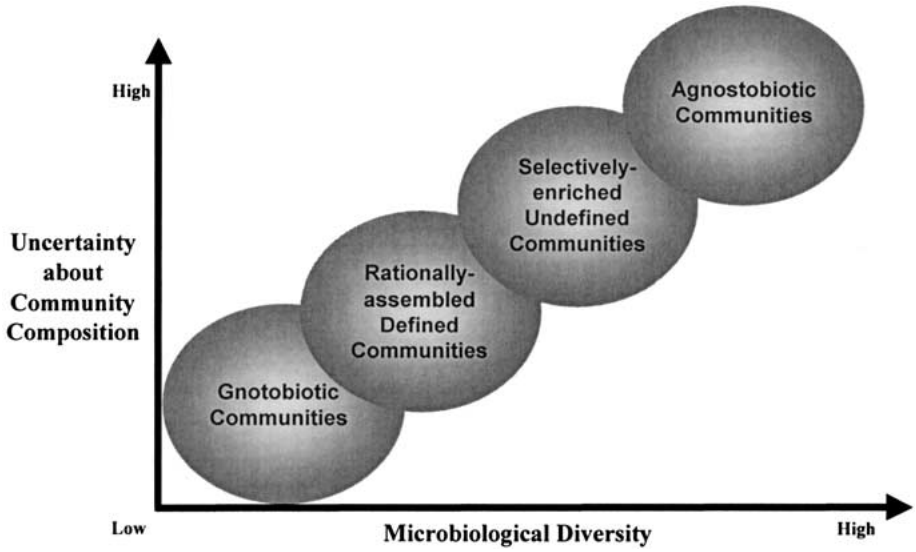

Figure 5. Conceptual approaches to microbial community engineering in closed bioregenerative systems.

a diversity treatment. Tests of this approach with rhizosphere communities found that the communities' susceptibility to invasion (one measure of system stability) was inversely related to diversity (Fig. 4). Gnotobiotic mixtures created from the pool of culturable types found in the original rhizosphere community were highly susceptible to invasion, suggesting that nonculturable types were important for conferring system stability.

Conclusions

The diversity of any community represents an equilibrium between the random invasion of new species and the random extinction of established species [57]. Spacecraft and ALS bioregenerative systems are unique, insular ecosystems characterized by relatively low biodiversity, elevated rates of extinction, and the potential for high rates of speciation by adaptive radiation events. Low diversity is correlated with increased variation in function [60, 64] and decreased resistance to invasion [30]. Microbial communities in ALS bioreactors and biomass production systems with low diversity are therefore subject to chaotic population fluctuations resulting in both loss of function and system productivity. Although a direct empirical relationship between diversity and function remains controversial [55], theoretical relationships have been proposed which equate the presence (diversity) and activities (function) of individual species within communities to the overall stability and productivity of the ecosystem [4, 64, 84]. Given that biological complexity is desirable because more diverse systems tend to be more productive and more stable, we must define predictors for ecosystem stability in space. Productivity is an easy parameter to define and measure within an ecosystem, but the measure of stability engenders far less confidence. At least four measures of ecosystem stability have been described: resistance, resilience, persistence, and variability [74]. Resistance and resilience measure the degree of displacement from equilibrium and time to return to equilibrium following perturbation, respectively. Persistence and variability measure two facets of diversity in the aftermath of disturbance, i.e., species richness and evenness. Since evenness is essentially unimportant for microorganisms because of the rapidity with which they respond to and alter their environment, resistance and resilience are the parameters of primary interest in microbial ecology. It is difficult at present, however, to assemble resilient biological systems given the methodological constraints of defining and capturing sufficient microbial diversity in a constructed microbial community, as shown in Fig. 5. Biological engineers and microbial ecologists must define the balance between the utilization of gnotobiotic (known) and agnostobiotic (unknown) microbial communities in space.

In both engineered and natural microbial ecosystems, diversity is beneficial because it contributes to degeneracy and an anthropocentric concept of microbial infallibility. The component functions of the system are buffered from failure by the presence of multiple ecotypes capable of performing an equivalent function. By design, however, closed spacecraft environments limit the pool of species that can be included to effect all necessary ecosystem functions. What research is required to resolve these conflicting interests in space exploration? Research that addresses the effect of the space environment upon the evolution of communities of microorganisms at scales ranging from the individual (e.g., genotype, phenotype, and mutation frequency) to the community (e.g., species richness, functional composition, stability, succession). It is not now possible to either understand or

predict the impact of biological diversity (whether functional, species, or genetic) upon ecosystem stability in the space environment. The population dynamics of any single microbial ecotype in an ALS system may be understood only in the context of interactions within the entire ecological community.

Microbial ecosystems respond to perturbations in complex ways in part because community assembly is a stochastic process: no single organism is able to outcompete all other organisms in all environments, and hence all the organisms within a particular functional group effectively have identical properties [19, 42, 47]. Although the members of the functional group may be interchangeable, subtle (i.e., at least to the observer) variations between members make it difficult to predict the effect of member substitution on ecosystem function. We have shown that biological diversity (species richness) and resistance to invasion are inversely related in ALS bioregenerative systems. This result leads to the conclusion that gnotobiotic approaches to community development for ALS bioregenerative systems will be unsuccessful. It remains unclear, however, whether the limitations of the gnotobiotic approach are due to the importance of nonculturable microbial types in the community or our ignorance of important community assembly steps. We have two factors in our favor for addressing these limitations in ALS bioregenerative systems. First, microbial evolution and community assembly occur on time scales that are accessible to empirical experimentation and modeling, even given the constraints of doing science in space. Second, a research platform for the necessary experiments exists. The International Space Station is an isolated microbial observatory. Although the ISS is not completely closed, the intake and release of material and energy are carefully accounted for, probably more so than in any controlled system on the earth's surface that approaches the complexity of ISS. Control and accountability are essential elements since the introduction of new organisms from outside can occur only at punctuated intervals, viz., visitation by a launch vehicle from earth. (Specifically, microorganisms are most likely to be brought aboard the station during visits by manned vehicles.) ISS represents the most realistic environment for the study of the evolution and development of microbial populations and communities in space. The experiment is under way, and changes in populations and communities will occur whether studied or not.

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