INTRODUCTION

A fundamental question in field-based studies of microbial communities is “what nutrient resource or environmental stressor is the limiting factor?” The concept of limiting factors goes back to Liebig’s Law of the Minimum in 1840, and takes into account not only the absolute amount of one resource but the relative ratios of all required nutrients (Sterner and Elser, 2002). With respect to nutrient resources, limitation may refer either to a constraint on the standing crop of biomass or on the specific growth rate of the microbes. Aquatic ecologists refer to cases in which a nutrient resource limits standing crop as under “bottom-up” control (Pfenninger and Amann, 2003). Usually, the concentration of limiting resource is reduced to a level where specific growth rate is less than the maximum. If loss factors such as predation or viral lysis maintain biomass levels below what nutrient resources could yield, the microbial populations are under “top-down” control, and their specific growth rate is probably near their potential maximum. If loss factors such as predation or viral lysis reduce the microbial populations to levels below what nutrient resources could produce, the microbes are under “top-down” control, and their biomass levels are maintained below what nutrient resources could yield, even if nutrient concentrations are reduced to levels where specific growth rate is near their potential maximum. If loss factors such as predation or viral lysis reduce the microbial populations to levels below what nutrient resources could produce, the microbes are under “top-down” control, and their biomass levels are maintained below what nutrient resources could yield, even if nutrient concentrations are reduced to levels where specific growth rate is near their potential maximum.

Determining the growth-limiting factor in natural habitats is analytically difficult. Under bottom-up control, the microbial population will reduce the in situ concentration of the limiting nutrient to low micromolar or nanomolar concentrations. Analysis of the growth-limiting factor or environmental stressors affecting microbes in situ is of fundamental importance but analytically difficult. Microbes can reduce in situ limiting nutrient concentrations to sub-micromolar levels, and contaminated ecosystems may contain multiple stressors. The patterns of gene or protein expression by microbes in nature can be used to infer growth limitations, because they are regulated in response to environmental conditions. Experimental studies under controlled conditions in the laboratory provide the physiological underpinnings for developing these physiological indicators. Although regulatory networks may differ among specific microbes, there are some broad principles that can be applied, related to limiting nutrient acquisition, resource allocation, and stress responses. As technologies for transcriptomics and proteomics mature, the capacity to apply these approaches to complex microbial communities will accelerate. Global proteomics has the particular advantage that it reflects expressed catalytic activities. Furthermore, the high mass accuracy of some proteomic approaches allows mapping back to specific microbial strains. For example, at the Rifle IFRC field site in Western Colorado, the physiological status of Fe(III)-reducing populations has been tracked over time. Members of a “subsurface clade” within the Geobacter predominated during carbon amendment to the subsurface environment. At the functional level, proteomic identifications produced inferences regarding (i) temporal changes in anabolism and catabolism of acetate, (ii) the onset of N2 fixation when N became limiting, and (iii) expression of phosphate transporters during periods of intense growth. The application of these approaches in situ can lead to discovery of novel physiological adaptations.

Keywords: physiological indicators, marine habitats, subsurface sediments
FIGURE 1 | Steps involved in the identification of physiological indicators and their application to natural microbial communities. A pure culture isolated from the environment is exposed to a range of physicochemical conditions hypothesized to be relevant to the in situ environment. Physiological responses specific to particular limitations or stressors are elucidated. Natural samples are then tested for the presence of these specific physiological indicators, to infer that the relevant limitations/stressors are acting in situ.

Identification and isolation of microorganisms in an environment

Laboratory experimentation with pure cultures under defined conditions

Understanding of microbial response to specific stresses in the laboratory

Application of this understanding to environmental samples

Identify (i) stresses affecting natural microbial populations, and (ii) microbial responses to these stresses

Increasing duration of experiment
not possible. The development of nucleic acid and proteomics sequencing technologies and analyses can generate the means to target specific, adaptive responses made by individual populations within a complex community.

THEORETICAL BASES FOR PHYSIOLOGICAL INDICATORS

There is an extensive literature from laboratory studies on pure cultures of diverse microbes, that demonstrate the regulatory responses in gene expression and protein activity that organisms make to specific nutrient limitations or stress factors (Tempest et al., 1983; Koch, 1997; Chung et al., 2006; Ferenci, 2008; Zhou et al., 2011). There can be significant differences in the molecular details and effects of regulatory mechanisms that have evolved in specific taxa, catabolite repression (Goerke and Stulke, 2008) represents but one example. However, it is also possible to extract some general principles that may be applied in developing targets for physiological indicators.

These principles have emerged both from empirical studies of microbes (particularly in continuous culture (Gottschal, 1990; Bull, 2010)) and from theoretical analyses of nutrient-limited microbial growth (Button, 1994; Koch, 1997).

The physiological adaptations to nutrient limitation can include the following:

1. Induction of general stress responses, such as heat shock proteins, that function as molecular chaperones to refold damaged proteins or phase shock proteins (Lund, 2009; Joly et al., 2010).

2. Detoxification mechanisms. A strategy broadly deployed across the microbial world is unidirectional efflux pumps – proteins that can transport toxic chemicals out of the cell. Although there are cases where pumps have specificity for particular toxicants, the so-called MDR (multidrug resistance) pumps have broad substrate ranges that extend beyond antibiotic efflux (Segura et al., 1999; Kruwitch et al., 2005; Higgins, 2007). In other cases, detoxification occurs in the cytoplasm, via reactions that metabolize the chemical to a less toxic form or synthesize metabolites that counter the toxic effects (Tam et al., 2006).

3. Alteration of cell composition (for example, by modifying the cell wall and membrane) in ways that reduce toxicity of the stressor. This involves changes in biosynthetic gene expression and metabolic fluxes (Nicolaou et al., 2010).

Recent work (Borden and Papoutsakis, 2007; Brynildsen and Liu, 2009; Alsaker et al., 2010; Rutherford et al., 2010) that has applied analysis of the transcriptome and proteome in response to stressors has observed responses in genes of unknown function – hence, there are many facets of stress responses in microbes whose biochemical basis are unknown.

APPLYING PHYSIOLOGICAL PRINCIPLES TO IN SITU MICROBIAL COMMUNITIES

The principles listed above have arisen from analyzing the physiological ecology of individual microbes. However, in a microbial community, the relative abundance of diagnostic genes, transcripts, or proteins for specific physiological processes can provide insights into the dominant metabolic processes in the community.

Lin et al. (2012) documented changes in the relative abundance of anaerobic respirers capable of using sulfate vs. nitrate as terminal electron acceptors across a vertical depth profile in Hanford subsurface sediments. Geochemical analyses demonstrated that the availability of those acceptors varied and quantitative PCR of dsrA and nosZ gene copies was related to these changes. The analysis (at the DNA level) indicated potential for a specific respiratory process; it would be more insightful to carry out analysis of gene or protein expression that would be related to actual physiological activity.

Identifying and measuring either the presence or shifts in abundance of proteins or gene transcripts that can be linked to specific physiological activities has emerged as a powerful tool to probe environmental stressors not detectable using other means. While metagenomic analysis of a microbial assemblage can offer insights into strain level community composition and functional potential, the coupling of a genomic foundation with either metatranscriptomics or metaproteomics has allowed expressed function to be interrogated. The first such integrated ‘omics approach utilized a combined metageneomics and proteomics technique, and focused on a microbial biofilm population within an acid mine drainage (AMD) system (Yvon et al., 2004; Ram et al., 2005). These studies allowed the effects of nitrogen limitation to be studied at the community level, rather than just for an individual microorganism. Metagenomic analysis indicated that
a low abundance strain, *Leptospirillum* group III was able to fix nitrogen, while the dominant biofilm community member, *Leptospirillum* group II, was unable to carry out this process. Likewise, community members *Ferroplasma* types I and II were similarly unable to fix nitrogen, but contained a large number of ammonia permeases and amino acid transporters, indicating an N scavenging role for these strains. Subsequent proteomic analyses confirmed the presence of Nif proteins that could be assigned to *Leptospirillum* group III, and ammonia uptake systems in *Leptospirillum* group II, consistent with this strain's inability to fix atmospheric nitrogen. In addition, this combined proteomic approach revealed mechanisms through which biomass was able to survive in metal-rich low pH AMD systems, utilizing a suite of chaperones, thredoxins and proteins involved in radical defense.

Similar techniques have subsequently been applied to a range of environmental systems. Here, we will focus on two environments where nutrient limitation and other stresses play a key role in determining microbial community structure and expressed functionality: oligotrophic ocean environments, and saturated subsurface aquifers.

**PLANKTONIC MARINE ENVIRONMENTS**

Survival and growth in oligotrophic oceanic surface waters poses a range of challenges for microorganisms due to the combined effects of nitrogen or phosphorous limitation plus exposure to intense light. With increasing depth, light irradiance decreases exponentially, and inorganic nutrient concentrations increase. Ammonia/ammonium is an important compound in both surface and deeper waters, while in deeper waters more oxidized forms of N such as nitrate and nitrate are available at higher concentrations. Numerous pure culture studies have investigated the mechanisms that allow the proliferation of biomass within these systems; this understanding has then been applied to the analysis of environmental 'omics datasets. Research involving ocean-dwelling *Prochlorococcus* strains in the lab has revealed niche-specific mechanisms for survival (Rocap et al., 2003), despite only varying 3% at the 16S rRNA level. *Prochlorococcus* strain MED4 is adapted for surface water conditions, while strain MET9313 is adapted for deeper waters by way of differing mechanisms for nitrogen usage, phosphorous uptake, and sensitivity to trace metals (Martiny et al., 2006; Toleman et al., 2006). The deep-water adapted strain MET9313 is able to utilize nitrate via a series of nitrite transport and reduction genes, whereas MED4 does not possess this metabolic capability (Moore et al., 2003). Using a transcript-based microarray approach, Martiny et al. (2006) demonstrated the expression of phosphorous-acquisition genes (such as components of the phe operon) under P-limitation conditions in strain MED4. At the proteome level, Lv et al. (2008) identified a correlation between the depth of water from which a *Prochlorococcus* strain had been isolated, and the atomic composition of the proteome of that strain. Strains isolated from deeper marine waters had higher nitrogen and sulfur contents than those from shallower waters, indicating long-term adaptation to environmental conditions. In spite of these long-term adaptations, Gilbert and Fagan (2010) hypothesized that fluctuating nutrient availability frequently encountered in surface waters favors mechanisms involving short-term down-regulation of nutrient-rich proteins. By comparing global mRNA transcripts from MED4 and MET9313 strains grown under a range of N and P conditions, it was shown that MED4 responded rapidly to nitrogen limitation by down-regulating nitrogen-rich ribosomal proteins. Contrastingly, the low-light, high-nutrient adapted MET9313 strain was shown to exhibit slow but sustained down-regulation of the same proteins.

The effects of sample depth in oceanic systems on microbial strategies for survival were recently investigated using a combined metagenomic and metatranscriptomic approach (Shi et al., 2011). Biomass from 25, 75, 125, and 300 m depths in the North Pacific was recovered, and revealed high expression of nitrogen metabolism genes (ammonium transporter, *amt*; dissimilatory nitrite reductase, *nirK*; urea transporter, *urt*; ammonia monooxygenase subunits, *amotABC*). The ubiquitous expression of *Prochlorococcus*-related *amt* and *urt* genes by *Prochlorococcus* populations was noted in contrast to the expression of the assimilatory nitrate reductase gene by only a small proportion of the population, consistent with previous observations of niche-differentiation within these microorganisms. Other environmental transcriptomic-based approaches have investigated the role of *N*$_2$-fixing diazotrophic cyanobacteria species in surface waters. In addition to detecting expression of the nitorgenase cluster, this strain was found to express the iron-stress-induced protein A (isnA) that is involved in protecting photosystem I from high-light-induced damage (Hewson et al., 2009).

The oligotrophic Sargasso Sea has proved a fertile environment for investigating microbial responses to nutrient limitations and environmental stresses; a metaproteomic study (Sowell et al., 2009) concluded that bacteria from the SAR11 clade dedicated a large proportion of their proteome (67% of total spectra) to transport functions, such as ABC transporters involved in substrate uptake. Given the low concentrations of P that can occur in the Sargasso Sea (<5 nm), the detection of the highly abundant periplasmic phosphate-binding protein PtxS and proteins involved in regulation of the *psb* operon (*PhoU, PhoR, and PhoB*) mirrored laboratory and field observations discussed earlier (Martiny et al., 2006). For SAR11 strains, a role for assimilation of inorganic nitrogen via ammonium, and incorporation into glutamine and glutamate was inferred from the detection of proteins involved in that pathway (*AmnR, GlnA, GlnT, and GltB*). Proteomic data indicated that *Synechococcus* were able to utilize a wider range of nitrogen sources including urea, nitrate, nitrite, and ammonium, whilst high-light-adapted *Prochlorococcus* strains preferentially used ammonium and organic sources of nitrogen, correlating with previously discussed transcriptomic data (Shi et al., 2011).

Finally, this study addressed some of the potential mechanisms with which those microorganisms in surface waters survive continual exposure to environmental stresses; the detection of chaperone proteins such as GroEL, GroES, and DnaK together with the membrane protease *HtrK*, at high abundances indicated a role for proteolysis and protein re-oligomerization in maintaining cell viability.
In other marine systems, Morris et al. (2010) carried out metatranscriptomic analyses of multiple biomass samples recovered from oligotrophic regions of the South Atlantic. Again, transport proteins for urac and ammonia were found to dominate the metatranscriptome in these regions, illustrating the importance of competing (Button, 1994) for the small concentrations of available N in these environments. In a contrasting nutrient-rich environment off the Oregon coast however (Sowell et al., 2011), no phosphate transporters were detected at high abundance, although the abundance of transporters for amino acids, taurine, and polyamines led the authors to hypothesize that carbon and nitrogen were more limiting in this system.

**SUBSURFACE SEDIMENTS**

Understanding the stress–responses and physiology of microbial communities in subsurface environments has long proved difficult. In situ measurements are hindered by the localization of biomass in small discrete pore networks within the subsurface. Despite these obstacles, at the Rifle Integrated Field Research Challenge (IFRC) site in Western Colorado, a series of biostimulation experiments via acetate amendment to the subsurface has allowed the study of microbial community responses to excess organic carbon. Given that target acetate concentrations during these experiments can range from 5 to 15 mM (Williams et al., 2009), other nutrient limitations may play a role constraining the specific growth rate of the indigenous microbial population. At the community level, a suite of molecular microbiological tools in numerous studies have indicated that Fe(III)-reducing bacteria are greatly enriched upon carbon addition to the subsurface (Konopka and Wilkins, 2010).

Water microbial community at the site revealed that the bacteria in situ acetate amendment. Tandem mass spectrometry data was searched against a database of predicted peptides from eight concatenated Geobacter genomes, and revealed the expression of proteins that had been predicted from previously described laboratory experiments such as NifD, NifH, and NifK. Measured ammonium concentrations in bulk groundwater samples were in excess of what has been reported as necessary for Geobacter growth (Mouser et al., 2009b). Instead, the expression of proteins involved in nitrogen fixation indicated that this energetically expensive process was occurring within the planktonic Geobacter population. Given that laboratory RNA and microarray studies (Holmes et al., 2004; Methe et al., 2005) had reported the rapid inverse response of nifD mRNA transcript levels to ammonium concentrations, this suggested that N limitation existed in pore spaces within the aquifer. Proteins involved in phosphate acquisition were less abundant across time points, suggesting less of a role for P limitation in controlling cell proliferation at the site.

Metal-stress responses typically induce the up-regulation of efflux pumps with which to remove toxic concentrations of metal ions that may build up within a cell wall. The Rifle IFRC site is located at a former uranium and vanadium milling operation, and a range of metals remain adsorbed to subsurface sediments. It is believed that some of these metals may be absorbed onto Fe(III) phases and subsequently mobilized as reductive dissolution of Fe(III) minerals occurs. Within metatranscriptomic datasets generated from Rifle IFRC groundwater biomass, heavy-metal efflux pumps have been detected, although the non-specific nature of the genome annotation limits the information that can be gleaned from these identifications (Wilkins et al., 2009). The more extreme highly acidic metal contaminated subsurface conditions at the Oak Ridge IFRC site have proved an ideal environment to investigate the community wide genomic potential for dealing with these stresses. Metagenomic analysis of the indigenous groundwater microbial community at the site revealed that the bacteria

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FIGURE 2 | The discovery of novel physiological responses via data mining of transcriptome or proteome data. Replicate environmental samples are collected along an environmental gradient where a measured physicochemical factor is known to vary. Multivariate statistical analyses of the molecular sequences and the associated environmental metadata identify genes or proteins whose abundance is related to environmental characteristics. Some of these may represent annotated genes that previously have been identified as physiological indicators. If the data mining suggests that there is an annotated gene that has not previously been recognized as part of the adaptive response, laboratory experiments should be undertaken to confirm its role under well-controlled conditions. However, the most powerful outcome of this approach may be the identification of unannotated genes that contribute to specific physiological adaptations. This generates a motivation for detailed functional genomic analysis, and subsequent biochemical experimentation to elucidate its novel function.
had evolved a range of mechanisms for existing in that environment (Hemmle et al., 2010). Nearly 150 genes were identified as being involved in heavy-metal resistance systems (heavy-metal translocating ATPases, mer operon, Co^{2+}/Zn^{2+}/Cd^{2+} efflux), while proton and small ion transport between the cytoplasm and periplasm was suggested as a mechanism for tolerating pH stress.

**FUTURE DIRECTIONS**

Understanding the environmental factors limiting microbial growth in nature has presented a range of problems that remain more than 30 years after they were articulated (Healey and Hendriks, 1980; Tempest et al., 1983). Although the introduction of molecular tools into microbial ecology over the past 20 years has illuminated the diversity of complex microbial communities, the more recent application of ‘omics technologies requires further refinements over the examples presented here in order to comprehensively reveal the limiting nutrient or environmental stressors impacting natural populations. With an appropriate sampling design and method of data analysis, these tools may be able to go beyond the transfer of laboratory-generated insights to field populations, and uncover novel mechanisms whereby microorganisms survive and/or proliferate under stressful environmental conditions (Figure 2).

The advances we foresee in this field by the further application of ‘omics techniques will be consequences of innovations in technology, computational analysis, and the application of sophisticated experimental design coupled to novel data mining approaches.

Technological advances in mass spectrometry should allow improved sensitivity and quantitation with regards to proteomic measurements, resulting in “deeper” analysis of samples, while newer peptide-specific techniques such as Multiple Reaction Monitoring (MRM) may be utilized for tracking the absolute abundances of specific biomarker peptides indicative of a particular stress in an environment (Wirrier et al., 2009).

Methodologies for nucleic acid sequencing have undergone dramatic changes that have reduced their cost by a factor of 50,000 over the past 10 years. However, the recent advances have come with an associated “cost,” i.e., a reduction in read length to a small fraction of a gene’s worth of information. The fundamental unit for analysis relevant to microbial ecology is not the base pair but rather the gene, and greater ecological insights regarding genes of unknown function can be made by genome context analysis, as genes of similar function are often closely linked in Bacteria (Morales and Holben, 2011).

Advances in computational methods may yield the extraction and reconstruction of complete or almost-complete bacterial genomes from complex environmental datasets (Ivenson et al., 2012), and we anticipate that the future generations of nucleic acid sequencing machines will yield increasingly longer read lengths that may span multiple genes and thereby ease genome context analysis.

The iteration between laboratory experiments under specific environmental conditions to identify physiological responses and their application to natural samples to interpret limiting conditions will continue to be important. However, future improvements in ‘omics technology and analysis can provide the opportunity to extend beyond this current formula, to the discovery of novel stress resistance mechanisms by comparative analysis of natural communities. Environmental genomists recognize that ecosystems contain “dark matter.” Although this term has been applied to sequencing the genomes of microbes that have not yet been cultivated, we believe that its deeper (and more challenging) meaning is the presence of significant proportions of functionally ambiguous or misannotated genes, as well as genes/proteins of unknown function, whether in uncultivated or cultivated microbes. Many metagenomic analyses have made use of much less than 50% of the sequence reads (Mackelprang et al., 2011); we suggest that this occurs because a high proportion of the remaining reads come from genes that encode proteins which play important roles in situ, but not under controlled conditions of pure culture. These traits may include physiological responses to nutrient limitation, stress, or intercellular communication.

Experimental strategies to discover novel or unrecognized functions from transcriptomic or proteomic data would be heavily dependent upon selection of natural systems and application of appropriate sampling design (Figure 2). For example, comparative spatial analysis (Anderson-Glenna et al., 2008; Kageyama et al., 2008; Lowell et al., 2009; Meyer-Dombard et al., 2011) across environmental gradients of stress factors such as salinity, temperature, heavy metals, or xenobiotics would generate correlations between environmental factors and the up- or down-regulation of genes or proteins. Statistical methodologies that identify genes comprising metabolic pathways within metagenomic datasets, and correlating these with environmental data have recently been described (Gianoulis et al., 2009). These would then become targets for deeper analysis of biochemical function. Techniques such as common garden or reciprocal transplant experiments that juxtapose environmental conditions (Reed and Martiny, 2007) can generate environmental forcing factors by which transcriptomics and proteomics may illuminate organism-specific responses.

The analysis of DNA sequences from environmental samples has generated tremendous insights into the membership and functional potential of microbial communities. Further maturation of transcriptome and proteome analyses will enhance our mechanistic understanding of the selective forces that are structuring those communities and the physiological responses made by specific organisms within them. With appropriate sampling designs and data mining techniques, we anticipate that these ‘omics techniques can discover unrecognized genes and proteins that perform critical roles in the ecology of microbes.

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