

WINOGRADSKY REVIEW

AMD biofilms: using model communities to study microbial evolution and ecological complexity in nature

Vincent J Deneff¹, Ryan S Mueller¹ and Jillian F Banfield^{1,2}

¹Department of Earth and Planetary Science, University of California, Berkeley, CA, USA; ²Department of Environmental Science, Policy, and Management, University of California, Berkeley, CA, USA

Similar to virtually all components of natural environments, microbial systems are inherently complex and dynamic. Advances in cultivation-independent molecular methods have provided a route to study microbial consortia in their natural surroundings and to begin resolving the community structure, dominant metabolic processes and inter-organism interactions. However, the utility of these methods generally scales inversely with community complexity. By applying genomics-enabled methods to the study of natural microbial communities with reduced levels of species richness, a relatively comprehensive understanding of the metabolic networks and evolutionary processes within these communities can be attained. In such well-defined model systems, it is also possible to link emergent ecological patterns to their molecular and evolutionary underpinnings, facilitating construction of predictive ecosystem models. In this study, we review over a decade of research on one such system—acid mine drainage biofilm communities. We discuss the value and limitations of tractable model microbial communities in developing molecular methods for microbial ecology and in uncovering principles that may explain behavior in more complex systems.

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Editor's note

Professor Jillian (Jill) Banfield was invited to contribute a Winogradsky review based on her recognized contributions to the field of microbial ecology using the acid mine drainage (AMD) biofilm as a model microbial community. During her studies of this defined community she and her co-workers established the new field of community proteogenomics, developed binning techniques for microbial community genome assemblies and recently determined the importance of CRISPR elements in shaping the community. Insight gained from the AMD biofilm community has led to increased understanding of how microbial populations evolve within communities and have established a paradigm for exploration of more complex microbial ecosystems.

Introduction

Model systems and the real world

Questions relating to microbial evolution and ecology are often approached using model organ-

isms (e.g. for studying the relationship between sequence divergence and sexual isolation (Roberts and Cohan, 1993) and model consortia (e.g., to study the balance between bottom-up and top-down controls on community structure (Bohannan and Lenski, 2000)). While these studies have been invaluable in furthering our understanding of the natural world, they capture neither the role of the uncultivated majority within communities nor the fact that (micro)organisms generally occur in communities made up of natural populations with inherent microdiversity (that is, not clonal populations; reviewed in Wilmes *et al.* (2009b)).

Studies of natural microbial consortia

Analysis of whole ecosystems with all of their attendant diversity is a hard problem. There has been progress toward addressing ecological and evolutionary questions *in situ* using marker gene-based culture-independent methods (Horner-Devine *et al.*, 2004). Despite some success, genomic heterogeneity between strains with nearly identical (Konstantinidis *et al.*, 2006) and identical (Simmons *et al.*, 2008) 16S rRNA genes complicates interpretation of marker-based patterns. Evidence that fine-scale heterogeneity is ecologically relevant in

Correspondence: JF Banfield, University of California, 369 McCone Hall, Berkeley, CA, 94720 USA.
E-mail: jbanfield@berkeley.edu

natural populations (for example, Hunt *et al.*, 2008) underlines the need to extend analyses toward the whole genome level. However, the complexity and poorly defined structural and functional boundaries of most natural microbial communities have precluded most ecological and evolutionary studies from achieving the level of detail possible for laboratory isolate-based model systems.

Clearly, systems with reduced complexity, either due to low species richness or dominance of one or a few populations, offer a special opportunity for analyses of community functioning. For over a decade, we have used molecular methods to study relatively low diversity biofilms that grow in the subsurface, where microbial activity stimulates pyrite (FeS₂) dissolution to form acidic, metal-rich solutions (Figure 1a). Such solutions form naturally when sulfides are exposed to air, water and microbial communities, and are often an undesirable effluent from mining sites (acid mine drainage, AMD).

AMD biofilms: a tractable model microbial ecosystem

Background

At our field site within the Richmond Mine at Iron Mountain in northern California, chemoautotrophic biofilms establish and mature at the air–solution interface of streams and pools that overlie sediment composed of quartz and sulfide (primarily pyrite) minerals (Figure 1b). Carbon fixation in AMD biofilms is driven by oxidation of iron released from the dissolving pyrite surface, which is coupled

to proton gradient-driven ATP formation. The by-product, ferric iron, reacts with pyrite surface sulfide/sulfur groups, stimulating further mineral dissolution (Figure 1a). In this environment, air, water and minerals sustain life, without measurable inputs from sunlight. Thus, the biofilms can be viewed as a relatively self-contained ecosystem in which there is tight and clearly defined coupling between inorganic and biological processes (Baker and Banfield, 2003; Druschel *et al.*, 2004) (Figures 1 and 2). Because microbial activity drives mineral dissolution, similar consortia are harnessed for bioleaching-based recovery of metals from sulfide phases (Bosecker, 1997).

It has been about 15 years since our group and collaborators first performed molecular studies on AMD communities that grow underground in the Richmond Mine. Initial 16S rRNA gene clone library and fluorescent *in situ* hybridization-based analyses revealed lower complexity than most natural systems (Figure 1c). We attribute the relatively low diversity, now evident at all taxonomic and trophic levels (Baker *et al.*, 2009), to the low solution pH (typically 0.3–1.2), high metal concentrations, and limited resource diversity. Low diversity and ability to resample defined structures and reproducible stages of biofilm succession over time (Wilmes *et al.*, 2009a) make AMD communities amenable targets for detailed cultivation-independent studies (Figure 1).

Many of the features contributing to the reduced complexity of AMD systems separate them from most other environments. This raises the question whether processes and interactions within this unusual growth environment are representative of

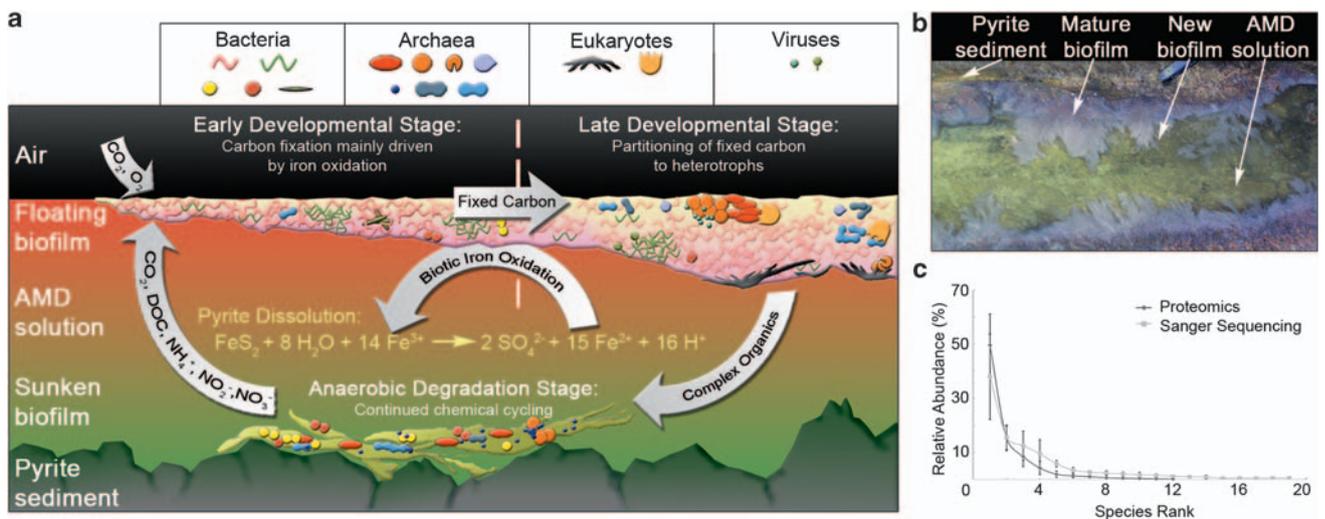


Figure 1 (a) Schematic illustrating important features that make the AMD system a good model for studying microbial communities (for example, relatively low species complexity, defined ecological succession patterns and trophic levels, tight biological–geochemical coupling, high biological productivity). (b) Picture of *in situ* biofilm developmental stages. (c) Rank–relative abundance curve for the AMD system communities. Sequencing reads from three Sanger sequencing libraries (~100 Mb each) that overlapped with a 16S rRNA gene were identified based on the best match in a database specific for the AMD system using BLAST. Proteomics data were analyzed by assigning each identified protein to the corresponding organism and calculating its relative representation as a fraction of all proteins identified. Error bars indicate the s.d. for each rank across samples. Although diversity is lower in the AMD system, the relationship is similar to various more complex systems (see for example Fuhrman *et al.*, 2008).

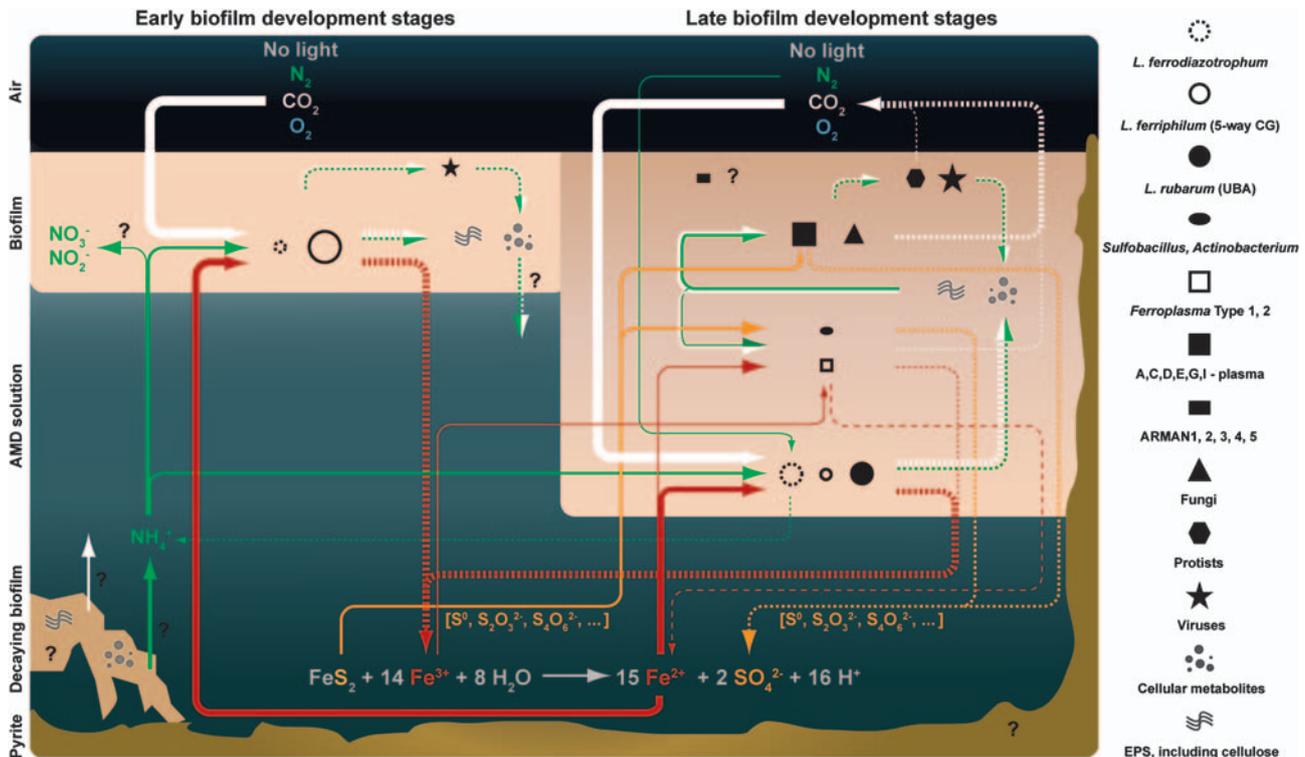


Figure 2 Ecosystem models beyond the ‘black box’. Combining insights from cultivation-dependent and -independent studies, metabolic roles can be assigned to the different members of the community (different symbols as per legend, symbol size indicates population size). Insights from population dynamics and proteomics data allow the determination of the relative roles of different populations (difference in thickness of the arrows, full lines = input; dotted lines = output). There is a division of metabolic roles as a function of biotic and abiotic environmental conditions. As an example, we here show shifts across different biofilm development stages. Parts of the nitrogen and sulfur cycles, the recycling of carbon after biofilm collapse and geochemical processes associated with the planktonic and sediment compartments still need to be linked to the specific populations involved. Quantifying fluxes of carbon and nitrogen through the system will be a focus of future research.

those that occur in other environments. For example, as discussed further below, reproducible patterns of community assembly are observed. This may distinguish the AMD system from others, since qits lower source diversity might drive these stable and predictable patterns (Curtis and Sloan, 2004). Nonetheless, evidence suggests community assembly in more diverse systems to be a non-random process, which can be predicted based on environmental parameters (Fuhrman *et al.*, 2006; Horner-Devine *et al.*, 2007). Moreover, despite conditions perceived by us as ‘extreme’, primary production rates in the AMD system are high, comparable on a $\text{gCm}^{-2}\text{y}^{-1}$ basis to rates for many terrestrial and ocean ecosystems (Belnap *et al.*, 2010). Rates are also similar on a per cell basis to those achieved by microbial primary production in the ocean (Belnap *et al.*, 2010). Interestingly, this implies that carbon fixation rates in $\sim 100\mu\text{m}$ thick biofilms are broadly equivalent to those achieved across the ocean photic zone. Comparable levels of ecosystem function across a very large range of species richness and physical scale increases our confidence that ecological principles inferred in the AMD system may predict patterns in more complex systems.

No model system can be expected to capture all features of other systems. Therefore, an important

question is whether basic biological processes differ fundamentally across ecosystem types. To answer this, one may take advantage of model system tractability to generate simple and clearly defined hypotheses to be tested across a range of environments. By understanding unique features of the systems where each hypothesis is tested, one can gain insight into conserved features across systems and into contingencies specific to sets of systems. This philosophy echoes abovementioned approaches used in model organism-based research. Moreover, new methods are almost certainly best devised and tested in less complex systems, thereby providing templates for approaches to interrogate more complex and less well-defined environments.

Early research: addressing biological questions while developing novel approaches

The first molecular studies in the Richmond Mine AMD system changed our thinking of which lineages were directly responsible for mineral dissolution at low pH (<1.5), switching focus from the relatively culturable *Acidithiobacillus ferrooxidans* (γ -Proteobacteria class) to various less easily cultivated *Leptospirillum* species (*Nitrospira* class) (Schrenk *et al.*, 1998). They also revealed the

importance of archaeal mixotrophs belonging to the *Thermoplasmata* class (Edwards *et al.*, 2000). Using 16S rRNA gene clone libraries and fluorescent *in situ* hybridization, these initial studies were also able to link aspects of community dynamics to environmental parameters. For example, seasonal drops in pH and increases in conductivity, resulting from the winter rain-driven displacement of highly concentrated AMD deep in the underground system, correlate to increases in the relative abundance of archaea (Edwards *et al.*, 1999; Bond *et al.*, 2000).

The ability to understand the physiological and ecological basis for spatiotemporal patterns in community structure is limited because many AMD community members are difficult to isolate. Arduous work to culture *Ferroplasma* and *Leptospirillum* species has classified these as mixotrophs and obligate chemolithoautotrophs, respectively (Johnson, 1998; Edwards *et al.*, 2000; Dopson *et al.*, 2004; Tyson *et al.*, 2005). The need for understanding the metabolic roles of all community members motivated the development of approaches to recover genomic information directly from the environment (Stein *et al.*, 1996). The comparatively low species richness of AMD biofilms made them ideal candidates for an effort to recover near-complete gene complements for all abundant organisms in a microbial community.

Community metagenomics

Using Sanger sequencing of a small-insert library (sequencing of both ends of ~3 kb inserts) 76 Mbp of data were acquired from a biofilm community from the Richmond Mine. These data were assembled to generate near-complete composite genomes for *Leptospirillum* Group II and a *Ferroplasma* type II population, as well as partial genomic data sets for three other populations (Tyson *et al.*, 2004). The *Leptospirillum* sequences provided the first genomic insights for a member of the *Nitrospira* phylum. The genomic inference that the *Leptospirillum* Group III population has the sole ability to fix N₂ in the community was used to isolate this organism, now described as *Leptospirillum ferrodiazotrophum* (Tyson *et al.*, 2005).

It is important to note that relatively deep sequence coverage (~6–25 times) enabled reconstruction of near-complete genomic representations of the dominant organisms in several AMD communities without reference to isolate genomes. In fact, using ~400 Mbp of Sanger sequencing data from multiple libraries (cumulatively, less than the equivalent of one plate of 454 titanium sequencing), it has been possible to recover ~12 near-complete bacterial and archaeal population genomic data sets (Tyson *et al.*, 2004; Lo *et al.*, 2007; Dick *et al.*, 2009). Several of these population genomic datasets have been analyzed to infer metabolic niches (Baker *et al.*, submitted; Goltsman *et al.*, 2009; Tyson *et al.*, 2004) (Figure 2). The most detailed annotation has been

performed for *Leptospirillum* Groups II and III, which allowed us to describe how these organisms fix carbon and generate energy through novel iron oxidation pathways (Goltsman *et al.*, 2009).

Insights into the biology of lineages that are unrepresented in current isolate-based genomic databases are the major advantage of community genomics methods. At first, it was unclear how broadly applicable these techniques would be for the reconstruction of population genomic data sets from natural communities in other environments. However, as evidenced by multiple studies since, the approach has been successful across a range of levels of system complexity, either when applied directly (Legault *et al.*, 2006; Martin *et al.*, 2006; Woyke *et al.*, 2006; Chivian *et al.*, 2008) or to molecular enrichments that focus on a segment of the whole community (Baker *et al.*, submitted; Hallam *et al.*, 2006; Pernthaler *et al.*, 2008).

Bacterial, archaeal and viral population metagenomics

Some of the most interesting findings from deep genomic sampling of AMD communities relate to evolutionary processes inferred from within-population genetic diversity. The dominant *Leptospirillum* Group II population displayed low polymorphism frequencies (0.08%), whereas a polymorphism rate of 2.2% was documented for the archaeal *Ferroplasma* Type II population (Tyson *et al.*, 2004). Closer inspection of the sequencing reads revealed that the archaeal population consisted of multiple strains with mosaic genomes resulting from extensive homologous recombination involving approximately three closely related but distinct sequence types (Tyson *et al.*, 2004). A detailed analysis of the *Ferroplasma* type I population revealed similar patterns. It was proposed that this process maintains diversity in the face of selection events, and thus confers increased population-level resilience to environmental perturbations (Allen *et al.*, 2007). Despite some skepticism about the initial results (DeLong, 2004), similar findings, mostly based on multilocus sequence typing, have emphasized the importance of recombination as an evolutionary process in other natural populations. These include findings for *Sulfolobus icelandicus* from hot springs (Whitaker *et al.*, 2005), *Halorubrum* in hypersaline environments (Papke *et al.*, 2004), and medically relevant organisms such as *Pneumococcus* (Hanage *et al.*, 2005).

Although deeply sampled community genomic data sets were clearly treasure troves for molecular studies, using them to answer evolutionary questions required new methods to visualize and analyze population genetic data. Eppley *et al.* (2007b) developed the program 'Strainer' for this purpose. Strainer was used to comprehensively analyze sequence variation and recombination patterns in two *Ferroplasma* populations. These authors showed that both intra- and interpopulation recombination

rates followed a log-linear relationship with sequence divergence (Eppley *et al.*, 2007a). Eppley *et al.* also documented recombination across the species boundary, possibly initiated within genomic regions that shared unusually high sequence identity. This is in line with a recent model involving temporal fragmentation of speciation (Retchless and Lawrence, 2007).

Allen *et al.* (2007) evaluated the form of population-level heterogeneity by comparison of environmental population sequencing reads with the genome of a *Ferroplasma acidarmanus* fer1 isolate originating from the same site some years earlier. The finding of high levels of within-population variation resulting from phage, plasmid and transposase insertion and deletion are substantiated by data from other systems (Coleman *et al.*, 2006; Cuadros-Orellana *et al.*, 2007; Rusch *et al.*, 2007).

Genome-wide determination of the rates of synonymous vs nonsynonymous polymorphisms in the dominant and deeply sampled bacterial population was used to evaluate evidence for neutral vs selective processes (Simmons *et al.*, 2008). On the basis of the absence of strong indications of positive selection for the maintenance of variation, the authors suggested a population natural history in which allopatric speciation was followed by migration into the same location and recombination (Figure 3). This evolutionary model is in line with inferences made previously for the archaeal populations in this system (Allen *et al.*, 2007).

Extremely high population-level diversity, nearing cell individuality, at the clustered regularly interspaced short palindromic repeat (CRISPR) locus, was an intriguing finding from two population genomic data sets for different *Leptospirillum* Group II strains (Tyson and Banfield, 2008) (Figure 3). The locus and associated proteins are now known to be involved in bacterial and archaeal defense against viruses and phage (Makarova *et al.*, 2006; Barrangou *et al.*, 2007; Brouns *et al.*, 2008). Comparison of the set of spacer loci (regions between the repeats transcribed as CRISPR RNAs) and the organization within and between the *Leptospirillum* populations indicated unidirectional locus expansion and provided evidence for lateral gene transfer of the locus. Notably, inferences about locus and virus–host interaction dynamics based on data from these natural populations are completely consistent with those from laboratory studies in which loci in *Streptococcus thermophilis* are activated by phage challenge (Barrangou *et al.*, 2007; Horvath *et al.*, 2008).

Given that spacers in the CRISPR locus derive directly from viral genomes, they could be used to identify and assemble viral genomes from the community genomic data sets (Andersson and Banfield, 2008). The deeply sampled virus population genomic data sets revealed extensive recombination among closely related virus types. Correspondence of the recombination block size to

the CRISPR spacer length for one AMD viral population suggests that viral recombination can result in evasion of the CRISPR-based host defense system (Andersson and Banfield, 2008). More recently, the CRISPR locus carried by plasmids in the AMD system was shown to target the protein machinery of the CRISPR locus of other plasmids (Goltsman *et al.*, 2009), indicating a broader role of the defense system (for example, in plasmid–plasmid competition). Such analyses have begun to bridge the gap between evolution and ecology by providing insight into the molecular dynamics resulting from predator/prey interactions, and by conclusively linking phage/viral populations with their hosts (Figure 3).

Metagenomic analyses of less abundant organisms in communities

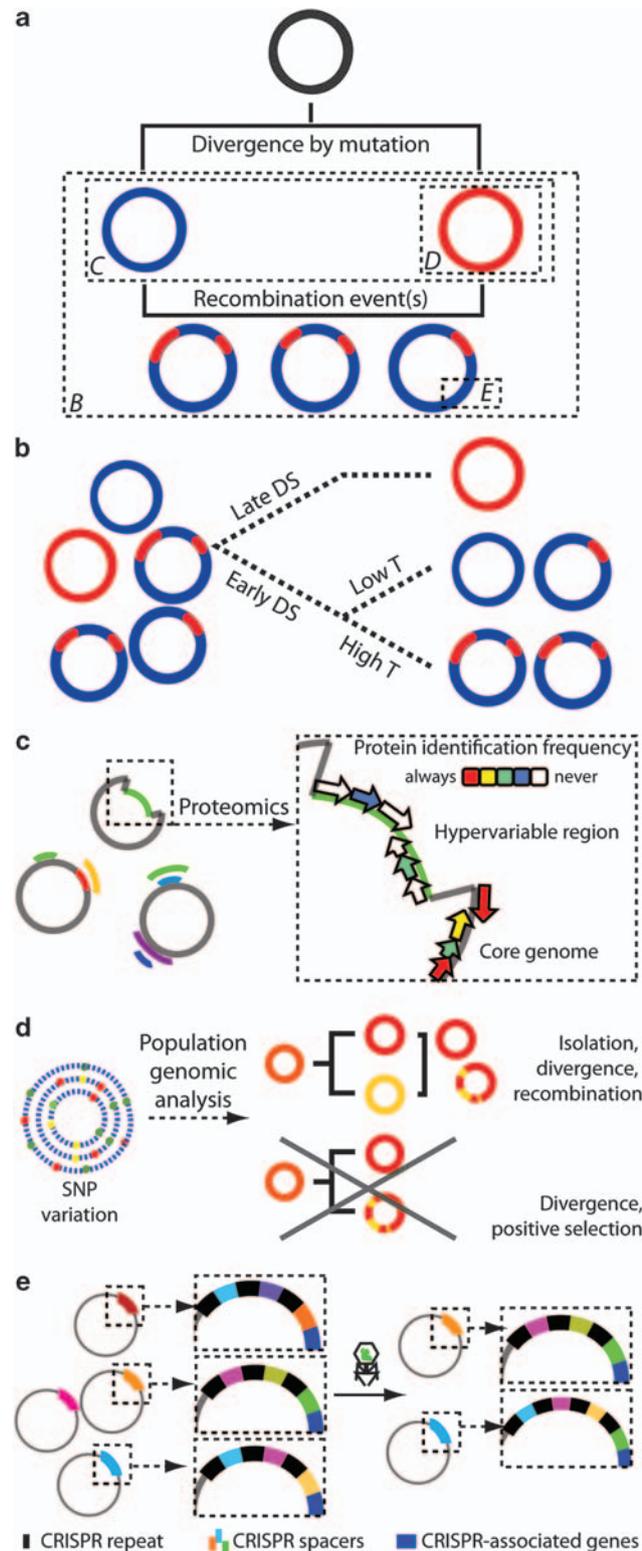
In obtaining a system-level understanding of ecological function in the AMD model system, most attention has been focused on the abundant organisms (comprising more than ~10% of the community), on the basis of the assumption that the majority of biological processes will be carried out by a few abundant populations (for example, paralleling the macroeconomic Pareto principle; Dejonghe *et al.*, 2001). Nevertheless, it has been shown that a relatively low-abundance organism (~0.3% of all cells) can carry out significant fractions of metabolic fluxes (Musat *et al.*, 2008). We have made progress with analysis of less abundant AMD community members. For example, Baker *et al.* (2006) discovered an enigmatic group of ultra-small archaea, represented by only a few sequence fragments, some of which contained rRNA genes <85% identical to any known sequence. Subsequent three-dimensional cryogenic electron tomography revealed that these tiny cells contain an average of only 92 ribosomes and have large internal tubular organelles of unknown function (Comolli *et al.*, 2009). Intriguingly, these community members seem to be highly targeted by viruses, and often by multiple morphotypes simultaneously. The combination of cryo-electron microscopy and targeted metagenomic analyses (Baker *et al.*, submitted) holds promise to unveil unexpected details of biology of low-abundance organisms.

The ability to interpret metagenomic sequence information to achieve ecological and evolutionary insights depends highly on methods to assign sequence fragments to organisms. Thus, we used curated composite genomes for nine archaeal, three bacterial and many virus populations, in combination with thousands of unassigned fragments from strain variants and low-abundance organisms to evaluate sequence-binning techniques. Dick *et al.* (2009) reported that a particularly effective method is based on emergent self-organizing maps (ESOM) of the tetranucleotide composition of genomic fragments (Teeling *et al.*, 2004; Abe *et al.*, 2005).

This study also provided insights into the sources of genome signatures that distinguish coexisting populations (Dick *et al.*, 2009). ESOM-based clustering revealed several clusters of genome fragments that, based on marker genes, could be assigned to specific low-abundance (percent-level) bacterial and archaeal populations. Despite the inability to assemble these

fragments into large genomic stretches, insights into the biology of the minor community members can be achieved based on the analysis of the binned gene inventories (Dick *et al.*, in preparation) (Figure 2).

Surveys of biofilm community composition from across many Richmond Mine environments, optimized by new genomic binning approaches, have documented ~20 predominant taxa, with each community usually dominated by only 4–6 taxa. We now know that AMD biofilm communities include representatives from at least two archaeal and eight bacterial divisions, as well as plasmids and viral (phage) populations (Andersson and Banfield, 2008). In addition, microscopic and 18S rRNA-based analyses have documented several fungal and protist species in mature biofilms (Baker and Banfield, 2003; Baker *et al.*, 2009). These results highlight the existence of multiple trophic levels with top-down predation, emergent patterns of organization (Denef *et al.*, 2010; Wilmes *et al.*, 2009a), succession, metabolic state switches on maturation (Mueller *et al.*, submitted) and ecosystem stability founded on diversity (Denef *et al.*, 2010) (Figures 1 and 2). These are the hallmarks of a complex biological system. For this reason, we conclude that the AMD system is not ‘simple’ (Handelsman *et al.*, 2007). The already apparent parallels with other environments (reviewed above) are strong indications that ecological and evolutionary insights from the AMD system will indeed be broadly applicable.



Functional analysis of microorganisms in communities through proteomics

Possession of a gene does not equate to gene function. Community proteomic analyses (also

Figure 3 Insights into links between evolutionary and ecological processes at different levels of resolution. **(a)** Although insights were derived for many archaeal and bacterial populations, this figure just focuses on the dominant *Leptospirillum* Group II bacteria. Two populations that derived from a common ancestor, as well as variants arising from recombination between them, are found in the Richmond Mine. Rectangles group the population(s) for which more details are shown in b–e. **(b)** The ecological distribution of the two populations and their recombinants primarily correlates with biofilm developmental stage (DS) and temperature (T). **(c)** Proteins encoded in hypervariable regions were rarely identified by proteomics, indicating that many have little or no role in environmental adaptation. Differential expression of shared genes is implicated in ecological divergence. **(d)** Population genomic analysis of single-nucleotide polymorphisms (SNPs) indicated variation within one population is not maintained by positive selection, but rather reflects allopatric divergence, migration and recombination, with some loss of variants. **(e)** Variation approaching the individual level is apparent in the clustered regularly interspaced short palindromic repeat (CRISPR) loci, which provide immunity against predation by specific viral/phage variants. Patterns of variation indicate that viral predation can shape population and community structure. Similar ‘hot spots’ of variability are found elsewhere in the genome, for example, at loci encoding cytochromes involved in Fe oxidation, raising questions about their potential role in fine-scale environmental adaptation.

referred to as environmental or metaproteomic analyses) provide a route to link genetic potential with activity. Community proteomic analysis was achieved with unprecedented depth by using the extensive set of gene predictions from an AMD biofilm to identify proteins through mass spectrometry-based methods capable of high mass accuracy measurements. In the initial study, Ram *et al.* (2005) extracted proteins from a biofilm similar to one for which genomic data were available, digested the proteins into peptides, separated the peptides by two-dimensional liquid chromatography, measured mass to charge ratio (m/z) values for peptides and their fragmentation products (high-throughput 'shotgun' proteomics) and identified >2000 proteins. This identified protein set comprised ~50% of the predicted proteins from the dominant organism, but only ~5% of proteins from the least abundant of the five genomically characterized members. Deep coverage of the proteomes of low-abundance members is often elusive, even in more recent analyses. Therefore, methodological advances, particularly those involving better protein/peptide separation methods (VerBerkmoes *et al.*, 2009a,b), will be essential for obtaining comprehensive proteomes from more diverse samples.

Despite current technical limitations, important insights have been gleaned from community proteomic studies. The first such study on the AMD system identified hundreds of proteins of unknown function that were previously classified as predicted proteins (Ram *et al.*, 2005). It is notable that several were located in genomic regions associated with mobile elements that, in general, do not have many proteins that are identified by mass spectrometry. However, some proteins of unknown function were very abundant, and were targeted for detailed analyses using cultivation-independent biochemical methods. This study led to the characterization of two cytochromes involved in a new iron oxidation pathway (Jeans *et al.*, 2008; Singer *et al.*, 2008). Visualization of newly identified cytochromes in intact biofilms revealed high concentrations of the protein only at the interface between the lower surface of the biofilm and the solution, indicating spatially heterogeneous activity levels at the tens of microns scale (Wilmes *et al.*, 2009a). Comparatively high levels of sequence variation have been documented in these cytochromes relative to the rest of the genome. This finding suggests that cytochrome sequence variation has a role in adaptation to redox gradients within the biofilms (Singer *et al.*, submitted; Jeans *et al.*, 2008; Singer *et al.*, 2008). This type of ecological differentiation based on cytochrome fine-tuning is supported by experimental studies of Springs *et al.* (2002), which detected large (~160 mV) changes in redox potential of cytochromes that differ by only a few amino-acid substitutions.

Proteomic data have been invaluable aids to metabolic interpretations based on genome reconstruction

(Goltsman *et al.*, 2009). For example, high abundances of pyruvate ferredoxin oxidoreductase and other functionally annotated proteins were used to deduce core metabolic pathways, including the route for carbon fixation, in *Leptospirillum* Groups II and III. Microscopy of intact biofilms showed that *Leptospirillum* Group II initiates biofilm colonization and that *Leptospirillum* Group III generally occurs as microcolonies or single cells dispersed throughout the biofilm (Wilmes *et al.*, 2009a). Observations such as the high numbers of sensory genes and abundant expression of these as well as motility proteins in this organism are consistent with these ecological patterns (Goltsman *et al.*, 2009) (Figure 2).

Ecological insights can also be achieved using proteomic analyses of large numbers of biofilm communities. For example, we have quantitatively analyzed protein-abundance patterns in specific organisms across a range of environmental (inorganic and biological) conditions. Notably, protein-abundance patterns of the dominant organism were most highly and significantly correlated with community composition, which is linked to changes in organismal membership over the course of ecological succession (Mueller *et al.*, submitted). In addition, abundances of proteins from less well represented populations correlated with inorganic parameters, suggesting distinct environmental niches for these members.

Strain-resolved proteomics

Shotgun community proteomics potentially can be used to distinguish expression patterns for closely related orthologous proteins to understand the importance of strain-level differentiation. A single amino-acid substitution in a peptide almost always changes the peptide mass (isoleucine/leucine being the exception), precluding peptide identification and reducing protein identification rates (especially for relatively low-abundance proteins). Thus, as the sequence of an organism in a sample diverges from the sequences in reference databases, protein identification is restricted (identification at <85% amino-acid identity is generally precluded) and abundance metrics become inaccurate (Deneff *et al.*, 2007). The high mass accuracy of current mass spectrometers allows for the discrimination of all peptides originating from closely related organisms (thus proteins), so long as sequences for alternative peptides are available. To take advantage of this capability, we have created databases of potential protein variants in the AMD system by teasing apart sequence variation that underlies the composite sequences in metagenomic data sets.

In several studies, we have distinguished single (Ram *et al.*, 2005) and groups of closely related candidate proteins in natural community samples (Lo *et al.*, 2007; Wilmes *et al.*, 2008; Deneff *et al.*, 2009). By performing this analysis at the peptide, protein and genome level, it is possible to infer the

genotype of a microbial population in a genomically uncharacterized sample. A first application of this method revealed that proteins encoded in some genomic regions matched one candidate genome type, whereas other regions matched a second, closely related genotype (~95% amino-acid identity). The approach, referred to as proteomics-inferred genome typing (PIGT), provided evidence for recombination involving sequence blocks of tens to hundreds of kilobases from two closely related *Leptospirillum* Group II populations (Lo *et al.*, 2007). PIGT on a more extensive sample set led to detection of multiple new genotypes comprised of different mixtures of the end member strain sequences (> 1000 proteins evaluated) and provided support for the notion that homologous recombination is used as a strategy for fine-scale environmental adaptation (Deneff *et al.*, 2009) (Figure 3). Very recently, the PIGT approach was expanded to carry out proteomic analyses of another system (*Geobacter*-dominated communities) for which metagenomic sequences were not available (Wilkins *et al.*, 2009).

The ability to detect recombination directly in the environment using a high-resolution, culture-independent method is closely linked to the ability to detect changes in protein-abundance levels, and thus activity levels, with strain-level resolution. Previous studies have focused on the role of gene content differences between related strains/ecotypes/species in ecological divergence (for example, Kettler *et al.*, 2007). In the AMD system, strain-resolved proteomics was applied to evaluate the relative roles of lateral gene transfer and changes in gene regulation in ecological divergence of two closely related populations (differing by 0.3% at the 16S rRNA gene level) (Figure 3). Proteins shared between populations were commonly identified and showed divergent expression levels, even when these populations co-occurred (Deneff *et al.*, 2010). Such findings illustrate that very closely related organisms can have distinct ecological roles, and indicate a key process in lineage divergence is proteome optimization, presumably driven by changes in genome regulation. The importance of looking beyond traditionally defined phylogenetic groups to understand ecological and evolutionary dynamics is currently being addressed in a variety of other systems as well (Hunt *et al.*, 2008; Wilmes *et al.*, 2008; Konstantinidis *et al.*, 2009).

Bringing complex biofilm consortia into the laboratory for hypothesis testing

Characterization of a series of environmental communities, as described above, is insightful by itself and is also crucial for the generation of hypotheses that can then be tested in controlled laboratory settings. The power to address biological questions using laboratory-grown communities derived from natural inocula is well established (for example,

Fernandez *et al.*, 1999). For the AMD system, we have developed methods to reproducibly grow multispecies biofilms in laboratory bioreactors. Proteomic methods have been key to optimizing growth conditions and verifying that communities are comparable at the functional and compositional levels with natural biofilms (Belnap *et al.*, 2010). An important advance in this study was achieved by mixing ¹⁵N-labeled laboratory-grown biofilms with the natural biofilms samples with which they were being compared. This allowed for relative quantification of peptides from laboratory-grown and natural communities using spectral peak areas detected in the mass spectrometry experiment. The ability to manipulate these laboratory-grown biofilms offers important experimental opportunities that are being exploited in ongoing research.

Future studies in the AMD model system

The overall tractability of the AMD system for molecular studies makes it a logical target for transcriptomic and metabolomic analyses, as well as studies that incorporate the roles of eukaryotes. But when such data are on hand, will it be possible to integrate metrics of activity and interaction into predictive models? There is no question that there are remaining barriers that need to be overcome before this goal is achieved in the AMD (and probably all other) systems. Among the most pressing of these is the gap in knowledge associated with the many proteins of unknown function. We hope that heterologous expression and subsequent biochemical screening of a large fraction of these, which have been targeted based on integration of metagenomic and proteomic data, can help to reveal their functions (Yakunin *et al.*, 2004; Kuznetsova *et al.*, 2005). Another challenge will be to define ecosystem indicators (for example, biomarkers in the form of metabolite, protein or transcript pools) that signify ecosystem state or the onset of a transition. Such methods can be developed for model systems such as AMD biofilms, and could ultimately find application in very complex environments where comprehensive analysis is neither affordable nor practical.

Scaling to more complex systems

It took 25 years to make the leap from the comprehensive sequencing of the first DNA virus, bacteriophage phi X174 (Sanger *et al.*, 1977) to the human genome (Lander *et al.*, 2001; Venter *et al.*, 2001), which is approximately one million times larger. Just 3 years after the first AMD metagenomic data set was published, the amount of sequencing attributed to a sample set had increased about 100-fold (Tyson *et al.*, 2004; Rusch *et al.*, 2007) and soon the factor will exceed 10 000 (for example, the Terragenome project, which aims at obtaining a comprehensive metagenomic data set from soil Vogel *et al.*, 2009).

The current expansion of sequencing capabilities clearly places almost any system within the reach of metagenomics (Wilmes *et al.*, 2009b). In addition, subcompartments of very complex systems can be analyzed using molecular enrichment or screening methods (Hallam *et al.*, 2006; Pernthaler *et al.*, 2008) and single cell genomics (Marcy *et al.*, 2007). Although there are some important issues regarding the accuracy of next-generation sequencing technologies, which currently limit their utility for population genetic analysis (Harismendy *et al.*, 2009), the combination of new technical developments and co-assembly of multiple data types should address this problem (Reinhardt *et al.*, 2009). Beyond this, the challenges with working with such vast data sets and complications due to the presence of many closely related genotypes are not yet fully appreciated. Bioinformatic tools and approaches, such as those being developed and refined through application to the AMD system, provide a starting point. However, it remains to be seen whether clear patterns and principles can be extracted from terragenome-scale efforts applied to complex, heterogeneous, and temporally varying environments. A limitation of the AMD system is that it may not anticipate the level of difficulty that will be encountered in this transition. However, progress in systems of intermediate (and even moderately high) diversity levels provide some encouragement that scaling of methods across complexity levels will be possible (Gill *et al.*, 2006).

At the functional level, continuing advances in proteomics (Sowell *et al.*, 2008; Verberkmoes *et al.*, 2009a,b), transcriptomics (Frias-Lopez *et al.*, 2008) and imaging techniques (Huang *et al.*, 2007; Behrens *et al.*, 2008) will provide deeper insights into ecological and evolutionary questions for systems that are currently only being characterized using phylogenetic markers (Cruz-Martinez *et al.*, 2009) or gene surveys (Tringe *et al.*, 2005). Integration of these methods with cultivation-dependent methods will further our understanding of microbial and global ecosystem functioning (Mou *et al.*, 2008).

Conclusion

The many features of the AMD system that render it particularly tractable for molecular-level, cultivation-independent analyses allow for identification of evolutionary mechanisms underlying strong and robust ecological patterns, despite the inherently noisy natural environment (Figure 1). Already, parallel findings in more complex systems are confirming the broader relevance of insights we have gathered regarding: (i) the character and ecological and functional roles of different forms of genomic diversity within and between populations (Figure 3); (ii) the interaction between bacteria, archaea, their phages and viruses (Figures 2 and 3); and (iii) relative rates of mutation and recombination (Figure 3). Such

insights significantly advance our understanding of microbial physiology in the environment and the driving factors of community assembly, while laying the groundwork for the development of predictive models of community composition and metabolism in natural settings (Figures 1 and 2).

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