



Engineering microbial consortia to enhance biomining and bioremediation

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In natural environments microorganisms commonly exist as communities of multiple species that are capable of performing more varied and complicated tasks than clonal populations. Synthetic biologists have engineered clonal populations with characteristics such as differentiation, memory, and pattern formation, which are usually associated with more complex multicellular organisms. The prospect of designing microbial communities has alluring possibilities for environmental, biomedical, and energy applications, and is likely to reveal insight into how natural microbial consortia function. Cell signaling and communication pathways between different species are likely to be key processes for designing novel functions in synthetic and natural consortia. Recent efforts to engineer synthetic microbial interactions will be reviewed here, with particular emphasis given to research with significance for industrial applications in the field of biomining and bioremediation of acid mine drainage.

Keywords: acid mine drainage, bioleaching, biomining, bioremediation, microbial consortia, synthetic biology, synthetic microbial consortia

INTRODUCTION

Natural microbial consortia are known to facilitate a wide range of complex tasks such as inter-species biofilm formation that allows microorganisms to persist in inhospitable environments (Keller and Surette, 2006). Syntrophic degradation of complex molecules also allows two species to complete metabolic reactions from which neither species would gain energy without the cooperation of the other (Zhou et al., 2011). Consortia play a crucial role in the human gut microbiome (Kau et al., 2011) and are known to heavily influence the ecological dynamics of the marine community (Giovannoni and Vergin, 2012). Humans have made use of natural consortia for millennia and selected them for better performance or desired properties in areas such as dairy processing (Reid, 2012), beer, and wine fermentation (Di Maio et al., 2012), and more recently in biogas processing (Lynd et al., 2002) and biomining (Rawlings, 2007).

The field of synthetic biology has developed a wide range of highly engineered clonal populations of bacteria to perform complex tasks such as differentiation (Süel et al., 2006), memory (Ham et al., 2008), counting (Friedland et al., 2009), and pattern formation (Liu et al., 2011a) as well as industrial applications such as production of antimalarial drug precursors (Ro et al., 2006), fuel like long-chain alcohols (Atsumi et al., 2008), and biosensors for arsenic in drinking water (Stocker et al., 2003). The construction and analysis of synthetic gene circuits has not only provided us with new tools for genetic engineering but has given deeper insight into naturally occurring gene circuits, their evolution, architectures, and properties as well (Sprinzak and Elowitz, 2005; Çağatay et al., 2009; Elowitz and Lim, 2010).

Considering that most synthetic circuits have been engineered in clonal populations it has been proposed that engineering synthetic consortia may allow for more complex tasks in industrial applications (Brenner et al., 2008; Sabra et al., 2010;

Momeni et al., 2011). Synthetic consortia may empower scientists and engineers to cultivate and make use of some of the 99% percent of microbes that have not been cultured yet and also to elucidate the role of the many genes that have yet unknown function(s), which might well be of particular relevance in microbial consortia (Wintermute and Silver, 2010). So far several proof-of-concept and industrial synthetic consortia have been engineered and reviewed (Brenner et al., 2008; Rollié et al., 2012); highlighted by a recent review focusing on engineered communication and biofuel processing (Shong et al., 2012).

The industrial practice of biomining (Olson et al., 2003) and bioremediation of heavy metal contaminations (Haferburg and Kothe, 2010) could potentially benefit from synthetic consortia as natural consortia have been shown to play crucial roles in these processes. This review focuses on using microbial isolates to construct consortia that would otherwise not be found together in Nature and discusses the potential use of genetically engineered species in bioremediation and biomining processes.

MICROBIAL CONSORTIA IN BIOMINING BIOLOGICAL PROCESSES IN METAL RECOVERY

Biomining entails the use of acidophilic microbes to facilitate the recovery process of metals from sulfide minerals in the processes of bioleaching and biooxidation. Biooxidation is the enrichment of metals, particularly gold, by mobilization and thus removal of interfering metal sulfides from ores bearing the precious metals (Rohwerder et al., 2003). Bioleaching is the solubilization of metals of interest such as cobalt, copper, and nickel from sulfide minerals. The two processes are industrially well established and are commercially applied worldwide (Rawlings, 2007). The microbes found in these environments are (extreme) acidophiles growing at a pH of 3 or lower and span a wide range of different phyla. The majority belong to the bacterial and archaea domains;

however, unicellular eukaryotes have also been reported (Baker and Banfield, 2003; Bonnefoy and Holmes, 2011).

The impact of microbial consortia in bioleaching, particularly in copper recovery, is widely recognized in literature and industry (Olson et al., 2003; Rohwerder et al., 2003). Microorganisms oxidize both sulfur and iron of sulfide minerals, such as pyrite. It is generally accepted that leaching takes place via an “indirect” mechanism, which can be divided into the “contact” and “non-contact” mode (Baker and Banfield, 2003; Rohwerder et al., 2003). The “indirect” mechanism assumes that chemoautotrophic iron-oxidizing microorganisms like *Acidithiobacillus ferrooxidans* or *Leptospirillum ferrooxidans* generate ferric ions by oxidation of ferrous iron (Rawlings, 2002). During the “non-contact” mode planktonic microbes oxidize aqueous ferrous ions to ferric ions, which in turn attack the mineral surface by chemical oxidation. The “contact” mode assumes a small reaction space between the microbial cell wall and the mineral surface where ferric ions are concentrated in biofilms for a localized attack of the sulfide mineral. Either mode yields different intermediate and final sulfur species depending on the ore leached. The thiosulfate mechanism applies for acid-insoluble metal sulfides such as molybdenite (MoS_2) and pyrite (FeS_2), and eventually yields sulfate (SO_4^{2-}) as the main end product. The so-called polysulfide mechanism applies for acid-soluble metal sulfides such as arsenopyrite (FeAsS) and chalcopyrite (CuFeS_2), eventually yielding elemental sulfur as the main end product (Rawlings, 2002; Rohwerder et al., 2003). Accumulating sulfur layers may act as a leaching inhibitor because of sterically impeding the ferric ion attack on the ore and thus affecting the growth of iron-oxidizing microbes. Chalcopyrite leaching is particularly sensitive to inactivation by formation of jarosite layers as a function of redox potential and is thus one of the most recalcitrant ore to leach (Viramontes-Gamboa et al., 2010). These sulfur layers however can be oxidized to soluble sulfate by sulfur-oxidizing bacteria such as *Acidithiobacillus caldus* or *Acidithiobacillus thiooxidans* (Dopson and Lindstrom, 1999; Sand et al., 2001; Mangold et al., 2011). Hence naturally occurring consortia of autotrophic iron-oxidizing microbes and sulfur-oxidizing microbes have been proposed to be symbiotic, potentially mutualistic (Rawlings et al., 1999) or at least synergetic in substrate use (Johnson, 1998; Roger Morin in Donati and Sand, 2007, 136).

COMMUNICATION IN NATURAL BIOMINING CONSORTIA

Research on extracellular polymeric substances (EPS) of *A. ferrooxidans* suggests that biofilm formation, which is crucial for the contact leaching mechanism, leads to an increase in redox potential and thus increase leaching rates as iron ions are trapped in the EPS (Sand and Gehrke, 2006). It is known that communication plays a major role in microbial biofilm formation (McDougald et al., 2012). The model bioleaching organism *A. ferrooxidans* produces and responds to compounds of the acyl homoserine lactones (AHLs) family used in auto-inducer 1 (AI-1) type quorum-sensing (QS) system (Keller and Surette, 2006) as well as to the c-di-GMP pathway (Hengge, 2009), which is also employed in QS. Two loci have been identified encoding for AHL synthases, the classical *LuxI*-type *afel* acyl synthase (Farah et al., 2005; Rivas et al., 2005) and *act* which is related to the LPA acyltransferase family

(Rivas et al., 2007). Both systems are involved in response to iron and sulfur substrates respectively, though *act* may be alternatively (or additionally) involved in cell membrane formation via fatty acid synthesis which is yet to be elucidated (Valdés et al., 2008). Recent studies also suggest a role of AHL-mediated QS in resistance toward high copper concentrations (Wenbin et al., 2011). The existence of a c-di-GMP pathway in *A. ferrooxidans* was discovered by analysis of its genome sequence (Ruiz et al., 2007). The pathway has been shown to respond to changes of the energetic substrate (iron and sulfur) as well as to the lifestyle of the bacteria (planktonic or biofilm-associated growth) by determination of intracellular c-di-GMP levels (Ruiz et al., 2011). Even though AHL type QS systems are absent from *A. caldus* and *A. thiooxidans* as inferred by genome analysis (Valdés et al., 2008), sequence analysis of the psychrotolerant *Acidithiobacillus ferrivorans* SS3 performed in our group (unpublished) suggests presence of an AHL type QS as we were able to identify an *act* homolog with 84% identity giving rise to the possibility for inter-species communication. Potential inter-species communication may also occur via the more common c-di-GMP QS. Related genes and the signaling compound were identified and isolated from *A. caldus* and *A. thiooxidans* (Castro et al., 2009). As biofilm formation is crucial for the leaching process it may be suitable to modulate AHL- and c-di-GMP levels to optimize attachment to ore particles for example, which may enhance bioleaching processes. However, although the existence of the above-mentioned pathways suggests that QS regulated biofilm formation plays a role in mineral solubilization, further experiments are required to prove this assumption.

CONSORTIA OF NATURALLY OCCURRING SPECIES

Whereas most studies have been performed on pure cultures of *A. ferrooxidans*, many early studies characterized mixed cultures of bioleaching organisms, mainly due to the difficulty in full separation of species in natural consortia (Harrison, 1984). Hence naturally occurring consortia have been characterized as well as defined consortia of naturally occurring organisms in order to elucidate mechanisms and synergies that improve the leaching process (Rawlings and Johnson, 2007). The impact of natural consortia was shown to be profound. In one example, a synergetic effect was observed by Qiu et al. (2005) during chalcopyrite leaching with a defined consortia of *A. ferrooxidans* and *A. thiooxidans*. The mixed culture was more efficient at leaching chalcopyrite than the pure cultures. The authors concluded that co-culture reduced the formation of inhibiting jarosite layers by the generation of sulfuric acid due to sulfur oxidation of *A. thiooxidans*.

Employment of heterotrophic acidophiles to remove inhibiting organic compounds that accumulate during growth led to acceleration of the leaching process. This was attributed to the increased growth rate of *A. ferrooxidans* while it was co-cultured with the heterotroph *Acidiphilium acidophilum* (Liu et al., 2011b).

Further examples for advantages of consortia are increased acid production (Okibe and Johnson, 2004), improved attachment to mineral surfaces (Noël et al., 2010), increased growth, and leaching rates (Bacelar-Nicolau and Johnson, 1999; Okibe and Johnson, 2004; Fu et al., 2008; Liu et al., 2011d; Naghavi et al., 2011).

While some of the above-mentioned consortia are most likely to occur in nature, engineering defined natural consortia has opened new possibilities for enhanced bioleaching (Rawlings and Johnson, 2007). Considering that researchers can choose from a wide range of microbes from different geographic locations, there is potential for additional, yet unexplored synergetic effects that may arise as these artificially assembled microbial consortia would not be encountered in nature. The use of consortia assembled from naturally occurring species is furthermore interesting because they would not be considered genetically modified and are hence not susceptible to regulatory procedures.

CONSORTIA OF NATURAL AND ENGINEERED SPECIES

To our best knowledge, hybrid consortia consisting of genetically engineered and naturally occurring bioleaching bacteria have not been reported so far. Even though some bioleaching organisms, in particular those of the *Acidithiobacillus* genus have been successfully transformed, genetic manipulations are difficult as transformation efficiencies are extremely low (Kusano et al., 1992; Peng et al., 1994). In fact only two knockouts (Liu et al., 2000; Wang et al., 2012) and two expression mutants have been reported in the scientific literature. One *rus* overexpressing *A. ferrooxidans* strain and another expressing the *mer* determinant for a mercury resistant *A. caldus* strain (Chen et al., 2011; Liu et al., 2011c). Once more suitable transformation protocols have been developed, it may be feasible to modulate QS signals with engineered microbes by either attenuating or amplifying natural signals or sending artificial signals to promote biofilm formation or mobilization respectively as recently demonstrated with engineered *E. coli* cells (Hong et al., 2012). Interestingly, it has been shown that once an initial consortia has been established, the power of evolution can be used to drive novel species interactions potentially resulting in increased consortia stability and productivity (Hansen et al., 2007).

Ultimately, engineered consortia could be deployed in industrial scale heap and tank leaching operations to improve bioleaching and biooxidation processes. However, as highlighted by Rawlings and Johnson (2007) it is crucial to consider that the tailored consortia have to compete with other microbes and their associated consortia in the non-sterile leaching environment. Usually this is not too much of a problem as the fastest growing species is usually the one leading to increased leaching. Depending on the ore leached, however, in particular chalcopyrite ores, high redox potential, which is associated with dominant iron-oxidizing microbes such as *A. ferrooxidans* or *L. ferrooxidans* is not appreciated as the continuous leaching process will stall after an initial high rate of recovery (Ohata et al., 2010).

Furthermore, hybrid consortia might be used to culture many of the yet uncultured microbes. There are abundant microbes in acid environments which are still to be cultured, characterized and their role in the ecosystem to be elucidated (Baker et al., 2010).

MICROBIAL CONSORTIA IN ACID MINE DRAINAGE BIOREMEDIATION

One major environmental consequence of industries such as mining, galvanic processing, and construction is the possibility of acid rock drainage (ARD) or in the particular case of mining,

acid mine drainage (AMD), where wastewater effluents and mine run-off are not properly managed. AMD may occur where access of oxidants to sulfide minerals, in particular pyrite, is facilitated due to mining operations as the surface area of the minerals is increased (Baker and Banfield, 2003). Both inorganic and biological reactions drive the acidification and heavy metal contamination of water due to oxidation of sulfide minerals (Ma and Banfield, 2011). The biological reactions account for the gross of AMD production with estimates as high as 75% (Baker and Banfield, 2003). The same organisms and consortia that are used in bio-mining operations are the major contributors to AMD generation, though AMD biofilms are mainly dominated by the chemoautotrophic Nitrospirae phylum bacteria *Leptospirillum* spp. (Gadd, 2009; Wilmes et al., 2009). AMD is dealt with usually in two ways, either by migration control or source control in case the axiom “prevention is better than cure” is feasible (Johnson and Hallberg, 2005; Das et al., 2009). In industrial environments AMD is usually managed at source by neutralization of wastewater and run-offs in rather costly abiotic neutralization processes with limestone. Therefore, the above-mentioned microbes and their biofilm environments are an interesting target for AMD management. Abiotic AMD mitigation control options as well as biological remediation processes have been recently reviewed (Johnson and Hallberg, 2005; Gadd, 2009) and are beyond the scope of this review. We will therefore focus on the few examples of biological AMD source control of AMD and discuss potential future applications.

Johnson et al. (2007) reported the use of heterotrophic acidophiles to colonize pyrite prior to exposure to iron-oxidizing bacteria to reduce dissolution of the mineral. The process, named “bioshrouding” was capable of decreasing the dissolution rate between 57 and 75%. This was presumably due to the heterotrophs’ biofilm that impeded attachment of the autotrophic iron-oxidizer. It remains to be seen if this approach is viable in industrial scale operations as iron-oxidizing autotrophs may out-compete the heterotrophs in the non-sterile environment though their growth could be impeded by the necessary supply of artificial carbon sources such as yeast extracts to foster the growth of the heterotrophs, which is known to inhibit growth of autotrophic organisms (Harrison, 1984).

Eukaryotic organisms such as algae and fungi have been reported in aquatic AMD environments, and could be engineered to mitigate AMD. Their natural role and potential application in bioremediation of AMD has been recently reviewed (Das et al., 2009). Engineering these eukaryotes for quenching of QS may be a very attractive option as resistance to quenching is unlikely to evolve (Defoirdt et al., 2010) and a broad range of quenching mechanisms are available. One example is the production of AI-1 type interfering halogenated furanones secreted by the algae *Delisea pulchra* (Czajkowski and Jafra, 2009). These small molecules mimic bacterial QS compounds and thus interfere with bacterial signaling and biofilm colonization.

Furthermore, the use of tailored bacteriophages and viruses (Lu and Collins, 2007) could potentially impede biofilm formation of autotrophs as naturally occurring viruses have been reported in AMD environments (Andersson and Banfield, 2008; Denef et al., 2010). Engineers employing this approach would have to

contend with bacterial defense mechanisms. The CRISPR (for Clustered Regularly Interspaced Short Palindromic Repeats) interference mechanisms are one such strategy bacteria and archaea use to evade phage treatment. Exogenous DNA is inactivated and processed to small elements of ~30 bp due to proteins encoded by the CRISPR-associated (*cas*) genes. These are then incorporated into the CRISPR locus from which they are constitutively expressed, processed and remain with an accompanying flanking region. The resulting CRISPR RNA (crRNA) binds to complementary RNA or DNA molecules and recruits Cas proteins to cleave the targeted nucleic acid depending on the organism (Marraffini and Sontheimer, 2010). This system has been used to rationally engineer crRNA-mediated mRNA cleavage in the extreme thermophilic archaea *Pyrococcus furiosus* using the native Cmr protein (Hale et al., 2012). Desired beneficial strains and consortia could therefore be potentially rendered “immune” by CRISPR engineering.

CONCLUSIONS AND FUTURE PERSPECTIVES

The ability to design and manipulate microbial consortia may allow biologists and engineers to enhance mineral recovery in bio-mining processes beyond the yields and productivities observed with naturally occurring consortia. Furthermore, it is likely that

synthetic consortia will also seep into other industrial sectors, which are currently mainly abiotic processes as microbial consortia are capable of more complex behaviors due to the combined properties of the individual organisms and the additional layers of regulation and adaptation to changing conditions.

The engineering of consortia will also be aided by the further characterization of the diverse species in bioleaching environments, which may have unique metabolic and physiological features. Prime candidates are the abundant archaea in biomining processes, which have not yet been subject to thorough research and are an untapped field of biological resources for industrial applications. The design and construction of synthetic and mixed microbial consortia will not only become a powerful tool in optimizing industrial processes but will also give us an insight into the evolution and emergence of naturally occurring microbial consortia. This will foster our understanding of higher-level system organization that is indispensable for designing complex functions.

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