

Comparative Genomic Analysis of Acidophilic Iron Oxidizing Bacteria from a Pilot Plant for the Microbial Remediation of AMD Water: Insights into Strategies for Speciation and Metabolic Adaptation to Life at Low pH and under Low Nutrient Concentration

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Abstract

Bacterial community analyses of samples from a pilot plant for the treatment of acid mine drainage (AMD) water from the lignite mining district in Lusatia (Germany) demonstrated the dominance of two groups of acidophilic iron oxidizers: a novel candidate genus "*Ferrovum*" and a group comprising *Gallionella*-like strains. Isolation of "*Ferrovum*" acidophilic strains as pure culture has proven difficult, though co-cultures consisting of "*Ferrovum*" and a strain of the heterotrophic acidophile *Acidiphilium* have been obtained. Similarly, *Gallionella*-like strains also proved recalcitrant to culture and isolation attempts resulted only in an enrichment culture comprising strains from several distinct taxa. Therefore, we employed a (meta)genomics approach to elucidate the metabolic potential of these microorganisms with the aim to deepen our knowledge of AMD bioremediation from lignite mines.

Introduction

Formation of acid mine drainage (AMD) is an environmental problem in many parts of the earth. The main cause of AMD formation is linked to anthropogenic mining activities during which sulfidic minerals become exposed to oxygen and water. This, in turn, leads to the oxidation of pyrite and, thus, the accumulation of sulfuric acid and the release of ferrous iron. This alteration to the environment creates an ecological niche for acidophilic bacteria that gain energy for their metabolic activity from the oxidation of ferrous iron to ferric iron. By doing this these acidophilic iron oxidising microorganisms enhance AMD formation because ferric iron represents a strong oxidant leading to further pyrite oxidation. However, these acidophilic iron oxidisers can also contribute to the remediation of AMD. For example, microbial iron oxidation is utilised in the 10-qm³ treatment plant Tzschelln that is located within the lignite open-pit lignite mine Nochten in Lusatia, Germany (Fig. 1). This process uses aeration of AMD water followed by ferrous iron oxidation by acidophilic iron oxidising microorganisms. The average hydraulic retention time of 8 h results in a constant production of acidity that maintains the pH within the treatment plant at approximately 3 (pH 2.85 – 3.1). As a consequence, the resulting ferric iron precipitates as the amorphous iron hydroxy sulfate mineral schwertmannite (Fe₁₆[O₁₆(OH)₁₀](SO₄)₃ • 10 H₂O; Bigham et al. 1990) which has various applications as a pigment or as a sorbent for the removal of arsenic from aqueous solutions (Janneck et al. 2010).

The bacterial diversity of the treatment plant has been investigated in a series of studies covering almost ten years because it is likely to play a fundamental role in the performance of the biotechnological process. This paper summarises findings from these studies and from recent genome analyses of representative strains of the abundant bacterial taxa within the treatment plant.



Figure 1 Treatment plant Tzschelln for the bioremediation of AMD water. The pilot plant is located at the lignite mine Nochten from which the AMD originates. Left: AMD within the oxidation basin of the pilot plant. Right: schwertmannite precipitated on carrier material within the treatment plant. (Photos: M. Mühling)

Methods

Isolation and culture of “*Ferrovum*” strains was attempted using the APPW medium (Tischler et al. 2013). Details on the isolation and maintenance of the mixed cultures of “*Ferrovum*” and *Acidiphilium* are provided elsewhere (Tischler et al. 2013; Ullrich et al. 2015, 2016a,b). Ferrous iron concentrations were quantified using the ferrozine method (Viollier et al., 2000). Enrichment of microaerophilic *Gallionella*-like acidophiles was based on that described by Kucera and Wolfe (1957), but conducted at acidic pH (3.5). Ferrous iron sulfide was prepared according to the method suggested by Emerson and Floyd (2005).

Details on the methods used to analyse the bacterial diversity within the pilot plant Tzschelln have been provided elsewhere (Heinzel et al. 2009a,b). These include, in essence, the preparation and sequence analysis of a clone library of PCR-amplified 16S rRNA gene fragments (Heinzel et al. 2009a) and the quantification of the abundant taxa *via* terminal restriction fragment length polymorphism (TRFLP) analysis and real-time quantitative PCR (Heinzel et al. 2009b).

TRFLP analysis of the mixed cultures was also used according to the description provided by Heinzel et al. (2009b) to quantify the distribution of “*Ferrovum*” and *Acidiphilium* in enrichment cultures.

Genomic DNA was isolated from “*Ferrovum*” and microaerophilic enrichment cultures using the MasterPure Gram Positive DNA Purification Kit (Epicentre) and the PowerSoil DNA Isolation kit (MoBio), respectively. Genome sequencing was carried out at the Göttingen Genomics Laboratory (G2L) using – in the cases of “*Ferrovum*” strains JA12 and PN-J185 – a hybrid approach using both the 454 GS-FLX Titanium XL system (Titanium GS70 chemistry, Roche Life Science) and the Genome Analyzer II (Illumina, 112-bp paired-end Illumina reads). “*Ferrovum*” strain Z-31 and the microaerophilic enrichment culture were sequenced employing only the Genome Analyzer II (Illumina, 112-bp paired-end Illumina reads).

Details on the sequencing output and the assembly and automated annotation of the sequence reads are provided elsewhere (Ullrich et al. 2016a,b). Methods employed for the comparative genome analysis of the “*Ferrovum*” strains are outlined in Ullrich et al. (2016b).

Digital DNA-DNA hybridisation (DDH) values were calculated for each pair of genomes using the Genome-to-Genome Distance Calculator (GGDC 2.0: <http://ggdc.dsmz.de/distcalc2.php>; Meier-Kolthoff et al. 2013).

Results

Analysis of the bacterial diversity of the AMD within the treatment plant revealed the dominance of strains whose 16S rRNA gene sequence showed highest similarity to that of two bacterial groups: the proposed species “*Ferrovum myxofaciens*” (Johnson et al. 2014) and the neutrophilic iron oxidiser *Gallionella ferruginea* (Heinzel et al. 2009a,b). Using a culture medium that simulates the chemical composition of the AMD within the pilot plant, it was possible to bring several “*Ferrovum*” strains into culture (Tischler et al. 2013). However, a detailed characterisation of these novel “*Ferrovum*” strains proved impossible because these cultures were consistently contaminated with a heterotrophic strain of the genus *Acidiphilium* sp. Therefore, we employed a two-tiered genomic approach in order to obtain insights into the metabolic potential of “*Ferrovum*” within the mixed cultures. This approach consisted, in essence, of the genome analysis of the contaminating *Acidiphilium* strain isolated from a mixed culture (Ullrich et al. 2015) and the subsequent (meta)genomic analysis of the same culture. Based on both datasets it was possible to reconstruct almost the complete genome sequence of a novel strain of “*Ferrovum*” that we termed strain JA12 (Ullrich et al. 2016a). Sequence analysis of two further distinct strains (PN-J185, Z-31) of “*Ferrovum*” from the pilot plant together with the available draft genome of the proposed type strain “*Ferrovum myxofaciens*” P3G (Moya-Beltrán et al. 2013) provided the basis for a first comparative genome analysis of the genus “*Ferrovum*”. Using a number of methods to calculate genomic distance between the four strains (e.g. genome-genome distance) strongly suggest that they belong to three distinct species: “*Ferrovum myxofaciens*” with the proposed type strain P3G and strain Z-31 and at least one further “*Ferrovum*” species represented by strains JA12 and PN-J185 (Ullrich et al. 2016b).

Furthermore, metabolic reconstruction of the annotated genomes of these strains revealed important insights into their metabolic potential and provided clues regarding their adaptation to low pH. For example, the presence of a urease encoding gene cluster in strains JA12 and PN-J185 indicates that urea hydrolysis is used to buffer against a potential inflow of protons (Mosler et al. 2013, Ullrich et al. 2016a). The role of urease in pH homeostasis has originally been shown for the gastric pathogen *Helicobacter pylori* (Eaton et al. 1991). Moreover, the absence of urease in type strain P3G and in strain Z-31 also provide an example for the species-specific metabolic traits within the genus “*Ferrovum*” (Ullrich et al. 2016b).

Attempts to isolate *Gallionella*-like acidophiles resulted in microaerophilic enrichment cultures (Fig. 2). 16S rRNA gene sequence based analysis of the bacterial diversity within one of these cultures indicated that it comprised at least three distinct *Gallionellaceae* strains that appear to be closely related to the neutrophilic iron oxidizer *Sideroxydans lithotrophicus* ES-1. The metagenomic data therefore proved to be particularly relevant in defining adaptive strategies to pH homeostasis since isolates belonging to the family *Gallionellaceae* are still restricted to the microaerophilic and neutrophilic iron oxidizers *Gallionella* and *Sideroxydans*. That is, the availability of the complete genome of strain ES-1 now permits the detailed comparison of the metabolic capacity of neutrophilic and acidophilic members of the genus *Sideroxydans* and, thus, the detection of biochemical features that are present in the acidophilic strains to support life under acidic conditions. For a start, the genome data of the acidophilic iron oxidizing strains indicate, similar to “*Ferrovum*” strains JA12 and PN-J185, the presence of a urease encoding gene cluster which is absent in the genome of *S. lithotrophicus* ES-1.

Moreover, metabolic reconstruction based on metagenomic data also indicates that nutrient requirements vary among taxa. This knowledge is of relevance for targeted improvements to the performance of the pilot plant. For example, the presence of genes possibly encoding proteins involved in phosphonate utilisation indicates that phosphonates may provide an alternative source of phosphorus to inorganic phosphate. However, experimental support is required to confirm this finding because not all proteins involved in phosphonate utilisation were detected within the metagenomic dataset.

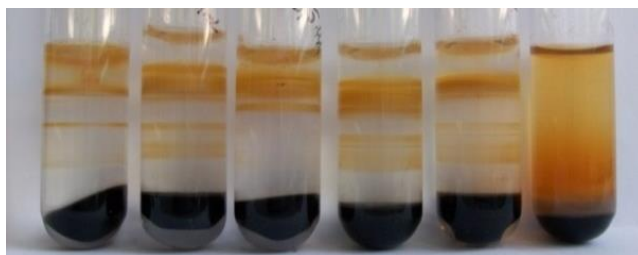


Figure 2 Microaerophilic enrichment cultures from samples of the inflow water into the pilot plant Tzschelln. The enrichment cultures were produced using the gradient tube technique of Wolfe and Kucera (1957) in combination with a novel medium found to favour the growth of acidophilic relatives of *Gallionella ferruginea* (Tischler et al. 2013). The last tube to the right is the abiotic control (no inoculum). The photograph was taken after 28 days of incubation under microaerobic conditions at room temperature. (Photo: A. Drechsel)

Conclusions

Based on the genome data obtained so far of these unique groups of acidophilic *Betaproteobacteria* it was possible to infer biochemical and phenotypic features which are difficult to determine *via* experimental approaches due to the lack of pure cultures. The comparative genome analyses of four “*Ferrovum*” strains were therefore the only means to reveal phenotypic features that seem to be common to acidophilic bacteria or unique to specific clades which, based on *in silico* determined genome distances, may be judged to represent at least two individual species within “*Ferrovum*”. Similarly, by assigning contigs to individual taxa using phylogenetic methods, it also proved possible to reveal phenotypic traits that seem to be responsible for the adaptation of strains of the genus *Sideroxydans* to life at acidic pH. Moreover, metabolic reconstruction from *Sideroxydans*-derived contigs indicates that acidophilic strains are able to utilise phosphonates similar to their neutrophilic relatives. Fertilisation of the AMD water with phosphonate-containing substances such as waste from paper and pulp or the textile industry may, therefore, result in an improvement to the performance of the treatment plant. Addition of phosphate to a simplified laboratory experimental simulation of the treatment plant already demonstrated a positive impact on ferrous iron oxidation rate (Tischler et al. 2014).

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