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Insights into the pathways of iron- and sulphur-oxidation, and biofilm formation from the chemolithotrophic acidophile *Acidithiobacillus ferrivorans* CF27

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Abstract

The iron-oxidizing acidithiobacilli cluster into at least four groups, three of which (*Acidithiobacillus ferrooxidans*, *Acidithiobacillus ferridurans* and *Acidithiobacillus ferrivorans*) have been designated as separate species. While these have many physiological traits in common, they differ in some phenotypic characteristics including motility, and pH and temperature minima. In contrast to *At. ferrooxidans* and *At. ferridurans*, all *At. ferrivorans* strains analysed to date possess the *iro* gene (encoding an iron oxidase) and, with the exception of strain CF27, the *rusB* gene encoding an iso-rusticyanin whose exact function is uncertain. Strain CF27 differs from other acidithiobacilli by its marked propensity to form macroscopic biofilms in liquid media. To identify the genetic determinants responsible for the oxidation of ferrous iron and sulphur and for the formation of extracellular polymeric substances, the genome of *At. ferrivorans* CF27 strain was sequenced and comparative genomic studies carried out with other *Acidithiobacillus* spp. Genetic disparities were detected that indicate possible differences in ferrous iron and RISC oxidation pathways among iron-oxidizing acidithiobacilli. In addition, strain CF27 is the only sequenced *Acidithiobacillus* spp. to possess genes involved in the biosynthesis of fucose, a sugar known to confer high thickening and flocculating properties to extracellular polymeric substances.

Keywords: *Acidithiobacillus ferrivorans*; Genome analysis; Iron oxidation; Sulphur oxidation; Biofilm; Extracellular polymeric substances

1. Introduction

Autotrophic acidophilic iron- and sulphur-oxidizing bacteria of the genus *Acidithiobacillus* catalyse the oxidative dissolution of sulfide minerals and play major roles in biomining and the genesis of acid mine drainage. They form at least four monophyletic groups [1], three of which (*Acidithiobacillus ferrooxidans* [22,38], *Acidithiobacillus ferrivorans* [17] and *Acidithiobacillus ferridurans* [19]) have been validated as distinct species. The main physiological differences between strains of *At. ferrooxidans* (and *At. ferridurans*, which display very similar traits) and *At. ferrivorans* are in their response to temperature and pH, and cell motility, and oxidation of hydrogen [17,20]. The pathways involved in the oxidation of ferrous iron (Fe(II)) and reduced inorganic sulphur compounds (RISCs) by *At. ferrooxidans* and *At. ferridurans* have been well studied [2,5–7,34]. In the case of *At.*

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ferrivorans, preliminary bioinformatic analysis of the genome of strain SS3 suggests a similar Fe(II) oxidation pathway but a less clear RISC oxidation pathway, with redundant genes from both *At. ferrooxidans* and *Acidithiobacillus caldus* present [28,29]. Previous data suggested that another Fe(II) oxidation pathway may have evolved in *At. ferrivorans*, since all the strains studied to date possess the *iro* gene [1] which encodes the periplasmic high potential iron–sulphur (HiPIP) protein Iro (for iron oxidase) that has been described as the first electron acceptor involved in Fe(II) oxidation in bacteria that were designated at the time as strains of *At. ferrooxidans*, but which are actually Group IV acidithiobacilli [11,15,25]. Furthermore, the *rusB* gene encoding rusticyanin B, an isoform of rusticyanin A which is known to play a key role in Fe(II) oxidation [2,5,6,34], was detected in all the strains of *At. ferrivorans* examined, apart from strain CF27 [1]. The kinetic rate constant for electron transfer between Fe(II) and RusB was shown to be approximately one half that of RusA [21] and its exact function in aerobic Fe(II) oxidation has not yet been clarified. It was also noted that strain CF27 often clustered away from the four other strains of *At. ferrivorans* studied by multilocus sequence analysis [1]. In addition, strain CF27 forms large macroscopic aggregates of mineral grains enmeshed with bacterial biomass in liquid media, suggesting a greater propensity for extracellular polymeric substances (EPS) biofilm formation than in other strains (Fig. 1).

To identify genetic determinants likely responsible for these physiological differences, the genome of *At. ferrivorans* CF27 was sequenced. Here we report the initial findings obtained from analysing and comparing the data with the genomes of other strains of *Acidithiobacillus* spp.

2. Materials and methods

2.1. Strains and cultivation conditions

Four strains of *At. ferrivorans* were used in the present study: NO-37^T (*At. ferrivorans*^{T}), Peru6, OP14, and CF27 [1].

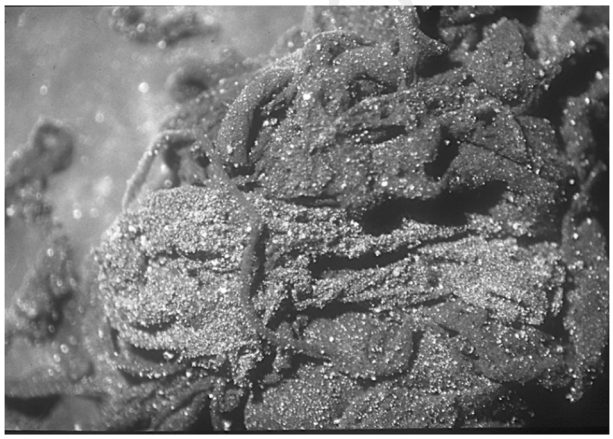

Fig. 1. Biofilm formation by *At. ferrivorans* CF27. Macroscopic image of biofilm growth of *At. ferrivorans* strain CF27, encapsulating fine grains of pyrite. The diameter of the image shown is ~5 mm.

These were grown routinely in a liquid medium containing 20 mM ferrous iron, basal salts and trace elements [29] at an initial pH of 1.9 (adjusted with sulphuric acid), at 30 °C. The type strains of *At. ferrooxidans* (ATCC 23270^T) and *At. ferridurans* (ATCC 33020^T) used in some experiments, were cultivated under the same conditions.

2.2. General DNA manipulations

DNA from *At. ferrivorans* strains NO-37^T, OP14, Peru6, and the type strains of *At. ferrooxidans* and *At. ferridurans*, was extracted from 5 mL of Fe(II)-grown cells to serve as a template in PCR reactions as described in Ref. [32]. As the primers previously used to amplify the *hip* and *rusA* genes in strains of *At. ferrooxidans* and *At. ferridurans* could not amplify these genes in strains of *At. ferrivorans* [1], new primer sets (Table S1) were designed based on alignments of the *hip* and *rusA* genes of *At. ferrooxidans*^{T} [40], *At. ferridurans*^{T} [8,3] and *At. ferrivorans* strains CF27 and SS3.

PCR were carried out in 20 µl reactions consisting of 1× GoTaq buffer (Promega, UK), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 25 pmol each primer and 1 U Hotstart Taq-Polymerase (Promega) using genomic DNA from *At. ferrivorans* NO-37^T, OP14, Peru6 as a template. The PCR program was as follows: initial denaturation at 95 °C for 5 min, 40 cycles of (i) denaturation for 30 s at 95 °C, (ii) annealing for 30 s at 58 °C for *rusA* and 62 °C for *hip*, and (iii) elongation for 30 s at 72 °C and a final elongation step of 5 min at 72 °C before the temperature was reduced to 4 °C. PCR products were purified using SureClean (Bioline Ltd., UK) and sequenced by Macrogen (Macrogen Inc., Korea).

2.3. DNA preparation, whole-genome sequencing, and genome analysis

In order to obtain sufficient DNA for genome sequencing, *At. ferrivorans* CF27 was grown in 1 L of 20 mM ferrous iron medium supplemented with 1.0% (w/v) elemental sulphur. Total genomic DNA was extracted from cells using Wizard Genomic DNA purification kits (Promega, UK). Whole-genome sequencing of *At. ferrivorans* CF27 was performed using Illumina technology. A mate-paired (MP) and paired-end (PE) libraries were created with 5 kbp and 330 bp insert size, respectively. Sequence data were then assembled using Velvet (http://www.ebi.ac.uk/~zerbino/velvet). To reduce the number of undetermined bases, GapCloser (http://soap.genomics.org.cn/soapdenovo.html) was performed onto the scaffold sequences with the PE reads. All general aspects of the library construction, sequencing and assembly were performed at Genoscope (www.genoscope.cns.fr, Evry, France). Computational prediction of coding sequences (CDS) and other genome features (RNA encoding genes, ribosome binding sites, signal sequences etc.), together with functional assignments were performed using the annotation pipeline implemented in the MicroScope platform [42]. The accession number of the CF27 draft genome is CCS020000001–CCCS020000082 (Bioproject PRJEB5721). Other genomes

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