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GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL ORGANISMS - ORIGINAL PAPER

Increased acid resistance of the archaeon, *Metallosphaera sedula* by adaptive laboratory evolution

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Abstract Extremely thermoacidophilic members of the Archaea such as the lithoautotroph, *Metallosphaera sedula*, are among the most acid resistant forms of life and are of great relevance in bioleaching. Here, adaptive laboratory evolution was used to enhance the acid resistance of this organism while genomics and transcriptomics were used in an effort to understand the molecular basis for this trait. Unlike the parental strain, the evolved derivative, *M. sedula* SARC-M1, grew well at pH of 0.90. Enargite (Cu_3AsS_4) bioleaching conducted at pH 1.20 demonstrated SARC-M1 leached 23.78 % more copper relative to the parental strain. Genome re-sequencing identified two mutations in SARC-M1 including a nonsynonymous mutation in Msed_0408 (an amino acid permease) and a deletion in pseudogene Msed_1517. Transcriptomic studies by RNA-seq of wild type and evolved strains at various low pH values demonstrated there was enhanced expression of genes in *M. sedula* SARC-M1 encoding membrane complexes and enzymes that extrude protons or that catalyze proton-consuming reactions. In addition, *M. sedula* SARC-M1 exhibited reduced expression of genes encoding enzymes that catalyze proton-generating reactions. These unique genomic and transcriptomic features support a model for increased acid resistance arising from enhanced control over cytoplasmic pH.

Keywords *Metallosphaera sedula* · Acidity resistance · Enargite bioleaching · Genome re-sequencing · RNA sequencing · Transcriptional responses

Introduction

The past few decades have witnessed the commercial application of bioleaching to extract copper from secondary copper sulfide ores [9, 61]. It is estimated that at least 15 % of current worldwide copper production was obtained by heap bioleaching [9]. Currently, processing of abundant yet refractory primary copper sulfide minerals, such as chalcopyrite and enargite is an area of active study [4, 10]. One major impediment for the application of bioleaching to process primary copper sulfide minerals is its low bioleaching rate by mesophiles [9]. Extreme thermoacidophiles provide an important alternative to achieve this goal because their use avoids mineral passivation that is the formation of surficial jarosite and sulfur that limits copper dissolution [4]. Due to the exothermic nature of copper sulfide mineral biooxidation, the temperature inside large ore heaps can reach 60–80 °C [6, 48]. This temperature inhibits bioleaching mesophiles and moderate thermoacidophiles but is suitable for extreme thermoacidophiles (optimal growth Tm $\geq$ 60 °C, pH $\leq$ 3).

The acidity of the leachate, an important physical parameter, affects the biodiversity of bioleaching microbial consortia and their biooxidation activities which in turn affects bioleaching rates and recovery of copper [25, 59, 68]. The acidity of the leachate derived from a bioleaching operation is determined by the following factors: (1)

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Content of acid-consuming gangue minerals, such as carbonate; (2) Composition and mineralogy of the copper sulfide ores (e.g., the proportion of acid-generating mineral sulfide such as pyrite; and the acid-consuming mineral sulfide such as chalcopyrite and pyrrhotite); (3) Reduced-sulfur-compounds oxidation activities of microbial consortia; (4) Weather conditions that would condense or dilute the leachate by way of evaporation or rainfall; and (5) The formation of secondary mineral ore phases that generate protons during bioleaching [7, 50]. A high concentration of Fe^{3+} in the leachate can result in formation of jarosite that precipitates on the surface of copper sulfide minerals at pH values up to 2, a process called passivation. Passivation inhibits dissolution of copper sulfide minerals. It is well known that in order to obtain high copper recovery yield in the bioleaching industry, it is necessary to maintain a high concentration of Fe^{3+}, the major bioleaching agent in the leachate, by reducing the pH of the leachate from pH 2 to 1 or even lower [26, 51].

Regulation of the pH within large heaps is technically not possible precluding a homogeneous environment during bioleaching. Instead the pH fluctuates due to unbalanced penetration of the leachate inside the heap together with the factors mentioned above [15, 64, 65]. The microniches inside the heap are spatially and temporally heterogenous with regards to acidity. Therefore, extreme thermoacidophiles that have a wider range of growth pH value, i.e., with higher acid resistance, should have a competitive advantage for bioleaching. Extreme thermoacidophiles belonging to the genera *Acidianus*, *Metallosphaera* and *Sulfolobus* were isolated originally from geothermal sites and have since achieved recognition for their utility in bioleaching [8, 13, 21, 27–29, 31, 32, 35, 40, 46, 47, 49, 55, 57, 66, 67] (see supplementary table S1). However, as some of these organisms grow poorly or not at all below pH 2.0, is it essential to improve this trait and assess its utility for potential application. As there are as yet no reports on the production and characterization of such organisms, it has been unclear whether increased acid resistance is a beneficial microbial trait in bioleaching. In this study, a more acid resistant derivative of wild type *M. sedula* named SARC-M1, was generated using adaptive laboratory evolution and used to test this possibility.

Materials and methods

Strains and cultivation

Strains used in this study included *Metallosphaera sedula* (DSM 5348T) (wild type) and the acid resistant derivative *M. sedula* SARC-M1. They were grown in basal salts medium (BSM) [2] as modified by Brock [11]. Complex medium (BSM) contained 0.2 % (w/v) tryptone adjusted to the indicated pH using sulfuric acid. The cultures were incubated at 75 °C in either glass screw-cap flasks with aeration in orbital baths or in glass screw-cap test tubes. Test tube cultures were placed in rotary drum agitators that were mounted in incubators with external DC motors [40]. Planktonic growth was monitored by light absorption at a wavelength of 540 nm using a Cary 50 spectrophotometer.

Isolation and screening of acid resistant *M. sedula* SARC-M1

Wild type *M. sedula* was inoculated from a frozen permanent into complex medium adjusted to a pH of 2.0 in a glass screw-cap test tube for heterotrophic growth. Mid-log phase cells (2×10^8) were sub-cultured into fresh complex medium adjusted to a pH of 1.50. This culture was passaged into fresh medium of increasing acidity in a repeated manner until growth was achieved at pH 0.90 (126 mM H^+). A clonal population was prepared using a solid complex medium adjusted to a pH of 3.0 consisting of 0.6 % (w/v) phytagel (Sigma, MO) that was incubated at 75 °C for 5 days.

Cultivation of strains

Wild type *M. sedula* and *M. sedula* SARC-M1 were grown heterotrophically to mid-exponential phase in complex medium adjusted to pH 2.0 and sub-cultured into media with different pH values ranging from 0. 92 to 3.0, respectively.

Mineral components

The composition of the enargite concentrate used in this study was enargite (60 %), pyrite (30 %), nowackiite (5 %) and quartz (5 %), as indicated by X-ray diffraction analyses. The main chemical composition of the concentrate is (w/w): 27.66 % Cu, 9.59 % As, 14.01 % Fe, 39.75 % S and 0.82 % Zn. The particle size of this concentrate was superfine and 84.1 % was less than 30 μm in diameter.

Bioleaching experiments

Enargite concentrate 0.5 % (w/v) was washed by incubating in 50 mL BSM medium at pH 2.0 and incubated at 75 °C with shaking (175 rpm) for 24 h. The enargite residue was then collected by centrifugation at 3,000 x g for 5 min then re-suspended in 50 mL of fresh BSM medium. Flasks were adjusted to pH 2.0 or pH 1.2, and an identical amount of wild type *M. sedula* or *M. sedula* SARC-M1 was inoculated into each flask at each pH value, respectively. Cultures were incubated at 75 °C with shaking (175 rpm).

Abiotic controls were included that were adjusted to identical pH values. Bioleaching leachate samples were sampled at intervals of 3-day increments.

Analysis methods

Planktonic cell numbers were determined using a Thoma counting chamber. The pH of the leachate samples was measured using a pH meter (Fisher Scientific, Model AB30) and the Eh values were determined using a platinum electrode with an Ag/AgCl reference electrode (American Marine Pinpoint ORP Monitor). The concentration of Fe^{2+} and Fe^{3+} ions was determined using the Ferrozine assay [53]. The concentration of Cu^{2+} was measured spectrophotometrically using diethyldithiocarbamate (DDTC) as an indicator at 440 nm [60].

Genome sequencing and RNA sequencing

Genomic DNA was isolated from *M. sedula* SARC-M1 as described previously [1]. RNA samples were extracted from mid-log phase wild type *M. sedula* grown at pH 2.00 and 1.50, and *M. sedula* SARC-M1 grown at pH 0.95. The purity and integrity of the DNA and RNA samples were confirmed by spectroscopic measurements (the ratio of absorbance at 260 nm to absorbance at 280 nm and the ratio of absorbance at 260 nm to absorbance at 230 nm) and confirmed by agarose gel electrophoresis. DNA and RNA libraries were prepared using DOE-JGI's automated process with a BioMek FX robot. The RNA samples were rRNA depleted using exonucleases (Epicenter mRNA-only prokaryotic RNA isolation kit) prior to the rest of the process. DNA and RNA samples were sheared using a Covaris E210 sonicator, followed by end repair and phosphorylation. Fragments ranging from 100 to 500 bp were selected for sequencing using an automated solid phase reversible immobilization selection system. Addition of 3′terminal adenine was made to the fragments followed by adaptor sequence ligation. RNA libraries with adaptors added were converted into cDNA libraries by reverse transcription. Genome and transcriptome sequencing of the libraries was done using an Illumina Hiseq 2500.

Sequences were mapped to the wild type *M. sedula* reference genome (NC_009440) using BOWTIE2 (ver 2.1.0) and SAMTOOLS (ver 1.0). Genome sequence information is available on NCBI. Mutations in the genome sequences that were located within open reading frames and identified through sequence comparisons were analyzed in more detail determining their codon positions and the effects they would have on protein sequence. The coordinates of each mutation were also cross-referenced to locations of the known domains in each protein to verify whether or not any mutations occurred within important functional domains. RNAseq read depth was evaluated across ORFs to eliminate artifacts and identify antisense transcripts and counts for each ORF were normalized using the RPKM method [45].

Nucleotide sequence accession numbers

The nucleotide sequences of this project have been deposited with GenBank under accession no. CP012176 for sequenced *M. sedula* SARC-M1; and RNA-Seq data are available in the Gene Expression Omnibus under the accession number GSE81414.

Results and discussion

Heterotrophic growth of wild type *M. sedula* and its SARC-M1 derivative

M. sedula SARC-M1 was isolated by adaptive laboratory evolution modified for use with extremophiles [41]. Repeated passage in media of increasing acidity followed by clonal isolation yielded a derivative lineage that could be stored and recovered without loss of its modified traits. To assess these traits, SARC-M1 was transferred from pH 0.92 to pH 2.0 and passaged twice. Wild type *M. sedula* was also passaged twice at pH 2.0. Both strains were then transferred to media at pH 2.0 and pH 0.92. Both strains exhibited similar patterns of growth at pH 2.0 (Fig. 1a). At pH 0.92 *M. sedula* SARC-M1 grew at a slow rate without a lag reaching stationary phase after prolonged incubation (207 h) (Fig. 1a). In contrast, no growth was observed for wild type *M. sedula* at this pH (Fig. 1a). To better assess the growth characteristics of these strains they were evaluated at pH values ranging from 0.92 to 3.0 (Fig. 1b). *M. sedula* SARC-M1 had a higher growth rate at pH values between 0.92 and 1.40, and from 2.30 to 3.20. In contrast, wild type *M. sedula* had a slightly higher growth rate at intermediate pH values ranging from 1.40 to 2.30. This suggests that *M. sedula* SARC-M1 would have a competitive advantage under bioleaching conditions where pH values fluctuate.

Enargite bioleaching capacity of *M. sedula* SARC-M1 and wild type *M. sedula*

The enargite bioleaching capacities of *M. sedula* SARC-M1 and wild type *M. sedula* were then compared at initial pH values of 2.0 and 1.2, respectively (Fig. 2). Only 15.83 and 19.05 % of available copper was solubilized from enargite for abiotic leaching samples. After 21 days of incubation at pH 2.0, *M. sedula* SARC-M1 and wild type *M. sedula* solubilized similar amounts of copper, respectively (60.63 and 64.07 %). Under more acidic conditions,

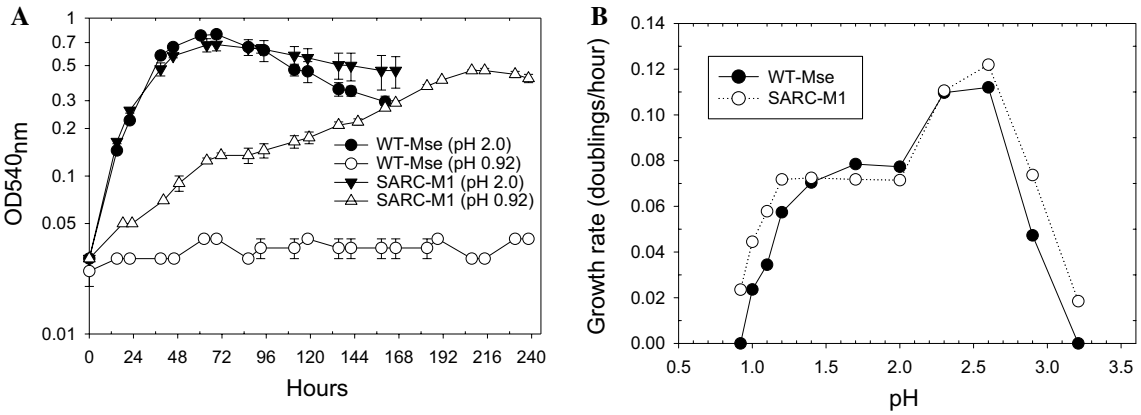

Fig. 1 Growth curve of *M. sedula* SARC-M1 (SARC-M1) and wild type *M. sedula* (WT-Mse) in complex medium with 0. 2 % (w/v) tryptone at initial pH 2.0 and 0.92 (**a**); and their growth rates at different initial pH values (**b**)

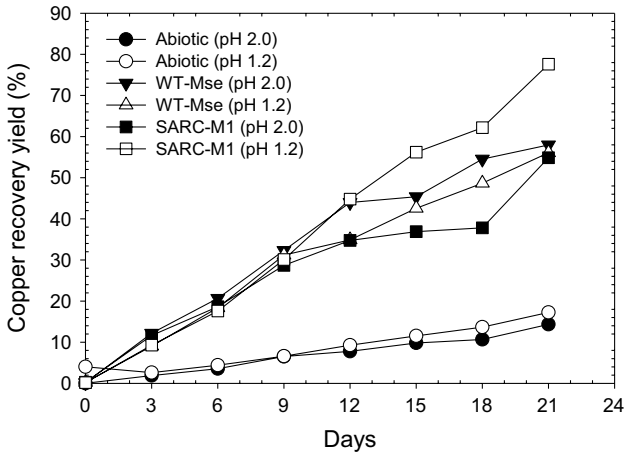

Fig. 2 Copper recovery yield of 0. 5 % (w/v) enargite bioleached by SARC-M1 and WT-Mse in complex medium with 0. 05 % (w/v) tryptone at initial pH 2.0 and 1.2, respectively

incompatible with the wild type strain, *M. sedula* SARC-M1 solubilized 23.78 % more copper than the wild type *M. sedula* (85.75 vs 61.97 %). The variation in pH value, Eh and total iron concentration in the leachate during enargite bioleaching was also determined (Fig. 3). The pH value of the leachate decreased gradually for both strains during incubation (Fig. 3a). At a starting medium pH of 2.0, the pH value of the wild type *M. sedula* culture was always lower than that of *M. sedula* SARC-M1 suggesting that its sulfur oxidation activity was greater. Conversely, at a starting pH value of 1.20, the pH of the *M. sedula* SARC-M1 culture was lower than that of wild type *M. sedula* again consistent with differences in sulfur oxidation activity. The abiotic control had a low Eh value of 340 mV (Ag/AgCl) during the bioleaching process (Fig. 3b). The Eh of the leachate decreased slightly during bioleaching under the higher acidity. Only traces of total iron were present in the leachate of the abiotic control at either pH values (2.0 or 1.20) indicating that enargite pyrite was resistant to acid dissolution (Fig. 3c). The total iron in the leachate of wild type *M. sedula* was always higher than that of *M. sedula* SARC-M1 at an initial pH of 2.0. This corresponded to a higher degree of copper solubility (Fig. 2). The total iron concentration in the leachate of wild type *M. sedula* and *M. sedula* SARC-M1 were comparable during enargite bioleaching at initial pH of 1. 20 (Fig. 3c), while the amount of solubilized copper by the wild type *M. sedula* was 23.78 % lower compared to that of *M. sedula* SARC-M1 (Fig. 2). This might be due to the sulfur oxidation capacity of wild type *M. sedula* suffering from some degree of acid inhibition as the pH of its culture supernatant was higher than that of *M. sedula* SARC-M1 (Fig. 3a). Perhaps elemental sulfur together with other inhibitory precipitates accumulate on the surface of enargite and inhibit further dissolution [56].

Genome sequencing of *M. sedula* SARC-M1

The basis for acid adaptation of SARC-M1 could be caused by mutations arising during successive passage and their identity might provide insight into the mechanism of acid resistance. To assess this possibility, the genome of the *M. sedula* SARC-M1 was determined and compared to the recently determined genome sequence of wild type *M. sedula* [40]. Based on this comparison, the genome of *M. sedula* SARC-M1 contained a total of four mutations in open reading frames and intergenic regions (Table 1). A tabulated summary of these mutations is presented with detailed information including mutation position in the genome, gene annotation, mutation type, gene/protein length and its proximity to identified domains (Table 2). Msed_0408 is annotated as an amino acid/polyamine/organocation transporter. A nonsynonymous mutation,

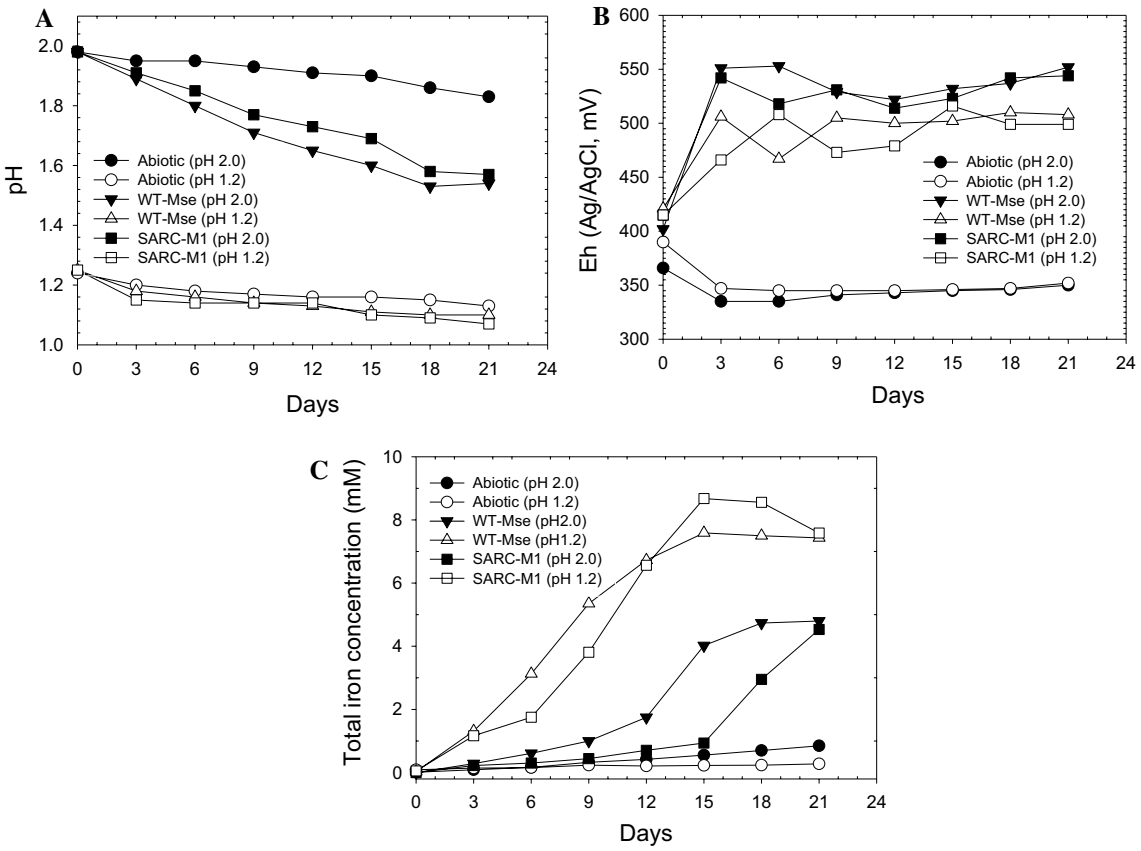

Fig. 3 pH (**a**), Eh (**b**), and total Fe concentration (**c**) of leachates during the bioleaching of 0. 5 % (w/v) enargite by SARC-M1 and WT-Mse in complex medium with 0. 05 % (w/v) tryptone at initial pH 2.0 and 1.2, respectively

Table 1 Summary of sequencing coverage and mutations

Strain name	Depth of coverage by Illumina reads	Substitutions in ORFs	Substitutions in intergenic regions	Reference
Wild type *M. sedula* DSM5348	1050-fold	24	3	[40]
M. sedula SARC-M1	143-fold	2*	2	This study

* No overlap with mutations detected in wild type *M. sedula*

Table 2 Mutation in acid-adapted *M. sedula* SARC-M 1

Genome coordinate	Substitution and description	Msed ORF	Gene function	Mutation location	Domain affected
360, 678	A → G, nonsynonymous (Ser → Pro)	0408	Amino acid/polyamine/organocation transporter	nt 952/1233; aa 318/410	Pot E
1,480,412	1 nt deletion	1517	Pseudogene	nt 812/854	

Ser318 to Pro318, was mapped to its Pot E domain. Previous studies in *E. coli* demonstrated that Pot E was a putrescine-ornithine antiporter that modulated intracellular pH homeostasis by consuming cytoplasmic protons under acidic stress [58]. Bioinformatic analysis indicated that this mutated site was located in a transmembrane helix resulting in its distortion; therefore, the function of Msed_0408 should be affected. A one nucleotide deletion also was found at position 812 in Msed_1517, previously annotated as a pseudogene but subsequently confirmed as the first archaeal PitA ortholog that regained function by insertion mutation [40]. The mutation at position 812 was unlikely to alter protein function because of the presence of several in frame stop codons in a promoter proximal location.

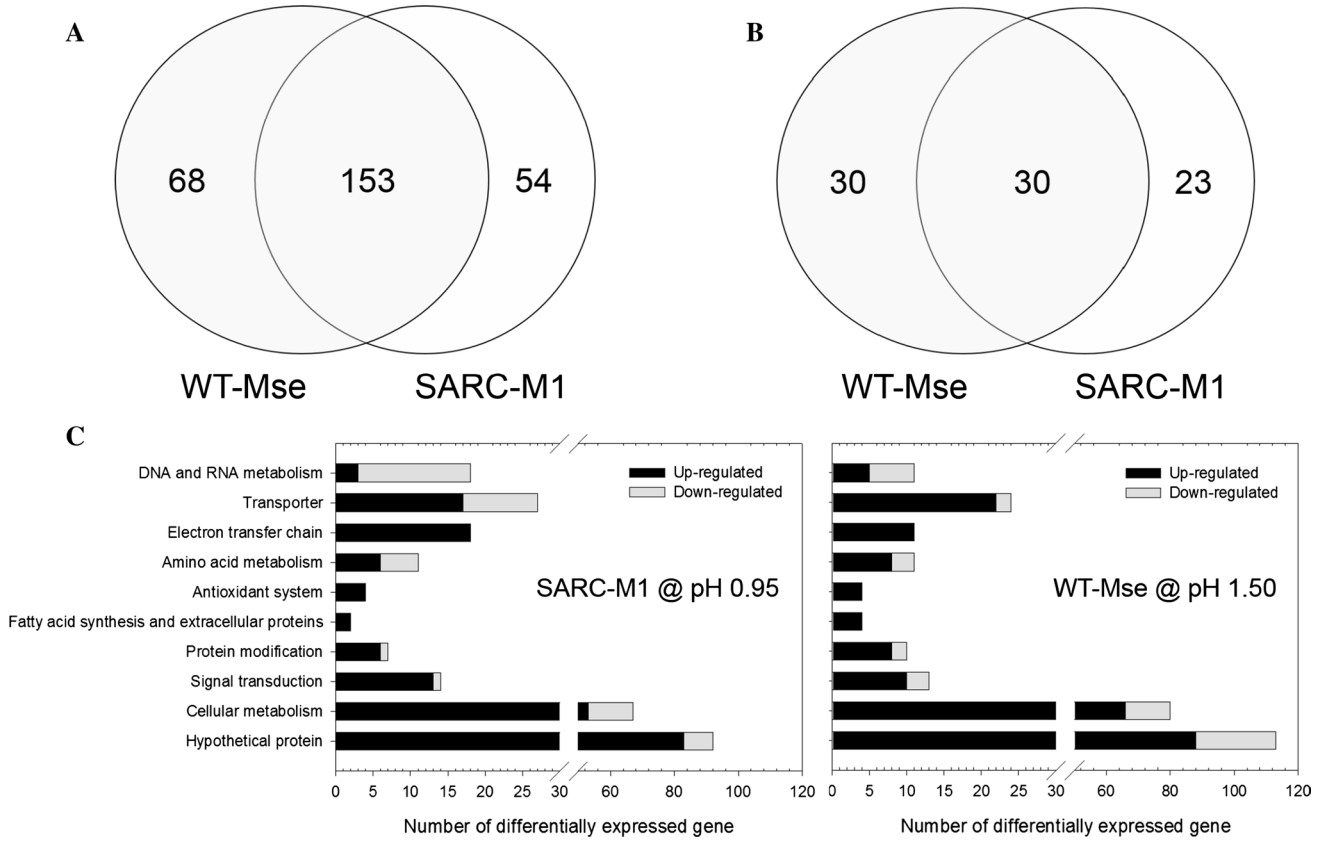

Fig. 4 Venn diagram of the up-regulated (**a**) and down-regulated (**b**) gene numbers (with >twofold changes) between pH 1.5-grown wild type *M. sedula* and pH 0.95-grown *M. sedula* SARC-M1. Transcriptomic profiles of pH 1.5-grown wild type *M. sedula* (**c**) and pH 0.95-grown *M. sedula* SARC-M1 (D) of genes with >twofold changes. Fold changes are relative to pH 2.00-grown wild type *M. sedula*

Transcriptomic response of *M. sedula* SARC-M1 and wild type *M. sedula* to acid stress

RNA-seq analysis with a read depth of 29 million to 32 million raw reads per transcriptome was conducted to examine changes in gene expression, with 98.86, 98.79, and 98.60 % of the raw reads for the transcriptome of wild type *M. sedula* grown at pH 2.00 and 1.50, and *M. sedula* SARC-M1 grown at pH 0.95, uniquely mapped to the genome. Compared to the transcriptome of wild type *M. sedula* grown at pH 2.0, 281 and 260 genes, respectively, with greater than twofold changes were identified in the transcriptome of wild type *M. sedula* grown at 1.50 and *M. sedula* SARC-M1 grown at pH 0.95 (supplementary table S2). Many of the ORFs that had expression changes in SARC M-1 (acid-adapted) grown at pH 0.95 also showed altered expression in wild type *M. sedula* grown at pH 1.5 (acid stress conditions). One hundred and fifty-three of those altered genes were up-regulated in both wild type *M. sedula* and *M. sedula* SARC-M1 (Fig. 4a), while only 30 genes were down-regulated in both transcriptomes (Fig. 4b). Ninety-one of the changes were ORFs for hypothetical proteins and proteins of unknown function in SARC M-1 gown at pH 0.95 (Fig. 4c). The remaining 169 affected ORFs that had annotated functions were sorted into 9 categories. These included cellular metabolism (66 ORFs), transporters (27 ORFs), electron transfer chain (ETC) (18 ORFs), DNA and RNA metabolism (18 ORFs), amino acid metabolism (11 ORFs), signal transduction (15 ORFs), protein modification (7 ORFs), fatty acid synthesis extracellular proteins (3 ORFs), and antioxidant system (4 ORFs) (Fig. 4c). The majority of ORFs with >twofold changes of expression in both transcriptomes included the genes for signal transduction and transcriptional regulation, antioxidant system, electron transfer chains, protein modification, cellular metabolism and hypothetical protein. This was consistent with the idea that grown at a lower pH imposed a common stress evident in both transcriptomes. In many cases, the changes in pH 1.50-grown wild type were of a lesser magnitude. This could indicate that the enhanced expression of these genes was essential for *M. sedula* SARC-M1 to survive in a more acidic environment.

Most of the differentially expressed genes that encoded proteins involved in signal transduction and transcriptional

regulation, were commonly observed in both transcriptomes with similar expression patterns (either up- or down-regulated). This pattern could represent induction of a genome-wide transcriptional response to extreme acidity and thereby constitute an acid stress-response regulon. In addition, this could partially explain why the majority of these genes with altered expression levels were shared in both transcriptomes. Msed_0486, Msed_0970 and Msed_1251 encode putative signal transduction proteins with CBS (cystathionine β-synthase) domains (Pfam: PF00571) and these may sense cell energy levels and other physiological pathways [5]. Msed_0715 encodes a PadR family (accession no. PfamPF03551) transcriptional repressor that might involve in regulating multi-drug resistance and detoxification [12]. Previous studies showed that the PadR family repressors LadR and LmrR, negatively regulated the expression the genes encoding the ABC-type multidrug resistance transporters in *Listeria monocytogenesis* and *Lactococcus lactis*, respectively [30, 69]. Therefore, the upregulated expression of Msed_0715 might lead to the downregulation of genes encoding the transporters. In addition, the regulatory protein ArsR, Msed_0717, was significantly up-regulated under acid stress (>fivefold). Msed_0717 might be involved in the regulation of genes encoding proteins relevant to acid stress responses since genes belonging to the ArsR family regulate genes with a diversity of physiological functions including regulation of heavy metal resistance genes/operons [44]. Msed_0892 and Msed_1126 encode a GntR family transcriptional regulator, which typically repress their target genes in the absence of their ligand [20]. Therefore, the up-regulation of Msed_1126 under acid stress could be responsible for the down-regulation of other genes, while the down-regulation of Msed_0892 could lead to the up-regulation of other genes. Msed_2209 encodes a transcription initiation factor IIB, which plays an essential role in pre-initiation complex assembly and transcription initiation by recruiting RNA polymerase II to the promoter [14]. The upregulated expression of this gene would lead to the upregulation of various genes across the genome.

Four genes encoding proteins belonging to protein integrity systems were upregulated in both transcriptomes. Msed_0242 encoding a membrane-bound heat-shock protein HtpX that probably participated in the proteolysis of misassembled and misfolded membrane proteins produced under extreme low pH. Previous study showed that the homolog of Msed_0242 in *E. coli* was involved in the proteolytic quality control of membrane proteins [52]. Msed_0640 encoded the heat-shock protein, Hsp20, and could protect the cell by preventing irreversible protein aggregation induced by the stress [34, 54]. Msed_1264 and Msed_1889 encoded ferritin and superoxide dismutase, respectively. Their upregulation could indicate they probably played an essential role in mitigating the toxicity of reactive oxygen species to cellular DNA and proteins under oxidative stress [19, 42, 62].

Nine genes encoding electron transfer chain components were commonly upregulated in both transcriptomes. Four genes (Msed_0321 to Msed_0324) encoding subunits of the membrane-bound SoxEFGHIM terminal oxidase complex [3] and 3 were genes (Msed_1428, Msed_1895 and Msed_1896) encoding subunit of NADH-ubiquinone oxidoreductase were upregulated. Their upregulation might indicate more energy was generated for energy-consuming metabolic processes. Two genes (Msed_1018 and Msed_1369) encoding peptidases were upregulated implicating a role in protein processing [18]. Msed_0593 and Msed_0820 encoding mechanosensitive ion channel proteins were upregulate and could alleviate excessive turgor pressure induced by extreme acidity. Their homolog in *E. coli* was activated by tension in the membrane and opened to relieve excess turgor generated under hypoosmotic shock [33, 37]. Most of the genes (65 ORFs) encoding hypothetical protein (or protein with function unknown) were also commonly expressed with >twofold changes in both transcriptomes.

Additional transcriptomic differences between transcriptomes included the following categories: DNA and RNA metabolism, transporter and electron transfer chain (Fig. 4c, d). These transcriptomic differences between SARC M-1 and wild type *M. sedula* provided insights into mechanisms required for the survival of SARC M-1 under extreme acidity. Fifteen ORFs involved in DNA and RNA metabolism were down-regulated in this strain. While only three of these ORFs were downregulated in the transcriptome of wild type *M. sedula* grown at pH 1.50. This could indicate modulation of nucleic acid metabolism under extreme acidity. There were nine transporters that were down-regulated in the acid-adapted strain SARC M-1 at pH 0.95 but not the acid-stressed wild type *M. sedula* at pH 1.50. These might improve acid adaptation if excess protons enter the cell through these. Seven more ORFs encoding electron transfer chain components were only upregulated in the transcriptome of *M. sedula* SARC-M1 grown at pH 0.95 and could indicate a requirement for increased energy required for proton extrusion under acid stress.

Difference in the expression of genes encoding proteins involved in signal transduction and transcriptional activation/repression were also observed in the two transcriptomes. Two transcriptional regulators, Msed_1209 and Msed_1351 were downregulated preferentially 5.00-fold and 3.32-fold, respectively, in the acid-stressed wild type at pH 1.50. However, four transcriptional regulators; Msed_0373, Msed_0832, Msed_1397 and Msed_1733, were only upregulated in the in acid-adapted SARC M-1 grown at pH 0.95.

Previous studies have shown that low pH stress promoted H^+ consumption and or extrusion thereby alleviating cytoplasmic proton excess in *E. coli*, *B. subtilis* and an acidophile PW2 [23, 38, 39, 63]. A similar pattern was also observed in the transcriptomes of both *M. sedula* strains grown at suboptimal pH, especially in the transcriptome of *M. sedula* SARC-M1 grown at pH 0.95 (Supplementary table S3). Genes encoding the electron transfer chains and membrane-bound enzymes or complexes that extrude H^+ out of the cytoplasm were upregulated. Some other genes encoding enzymes catalyzing H^+ consuming reactions in cytoplasm were also upregulated at low pH. While, genes encoding enzymes that catalyze H^+ generating reactions were down-regulated. The four genes (Msed_0321 to Msed_0324) encoding subunits of the membrane-bound SoxEFGHIM terminal oxidase complex [3] that involved in energy generation and H^+ extrusion were up-regulated under acid stress. The genes (Msed_1428 to Msed_1431 and Msed_1895 to Msed_1897) encoding subunits of NADH-ubiquinone oxidoreductase (complex I) were also up-regulated. This complex could absorb electrons from NAD (P)H, a by-product of acid-consuming reactions, and extrude H^+ out cytoplasm [16, 17]. Most of the down-regulated genes encoded enzymes that catalyze acid-generating reactions that employ ATP (with 4 ionizable protons) as a substrate and consequently generate ADP and P_i, both of which contain 3 ionizable H^+. The expression level of genes (Msed_1914 to Msed_1920) encoding the subunits of ATP synthase that couple ATP biosynthesis with the influx of H^+ remained constant under acid stress [24]. This indicated that ATP synthase did not induce extra proton load in the cytoplasm at the transcriptional level under extreme acidity. Therefore, down-regulation of these genes encoding these enzymes involved in catalyzing acid-generating reactions would decrease the proton load further in the cytoplasm of *M. sedula* SARC-M1 under extreme acidity. Twenty-six genes encoding electron transfer chain components as well as membrane-bound enzymes/complexes that extrude H^+ out of cytoplasm or encoding cytoplasm enzymes catalyzing H^+ generation/consumption reactions, were observed to be up- or downregulated in the transcriptome of wild type *M. sedula* grown at pH 1.50. In addition an additional 17 genes encoding proteins involved in H^+ homeostasis with >twofold expression changes were found exclusively in the transcriptome of acid-adapted *M. sedula* SARC-M1 grown at pH 0.95 (Supplementary table S3). This indicated that *M. sedula* SARC-M1 had a higher capacity to maintain H^+ homeostasis in the cytoplasm under extreme acidity. This could explain why *M. sedula* SARC-M1 grew at pH 0.92 while the wild type *M. sedula* could not (Fig. 1A). Genes (Msed_0289 to Msed_0291) encoding the subunits of the SoxABCL complex which translocates H^+ out of cytoplasm and simultaneously converting H^+ and oxygen to water in the cytoplasm were uniquely upregulated in the acid-adapted *M. sedula* SARC-M1 grown at pH 0.95 [22, 36]. More genes (Msed_1429 to Msed_1431, and Msed_1897) encoding the subunits of the complex I were upregulated in the transcriptome of *M. sedula* SARC-M1. Msed_1432 and Msed_1433 encode the membrane-bound homologous

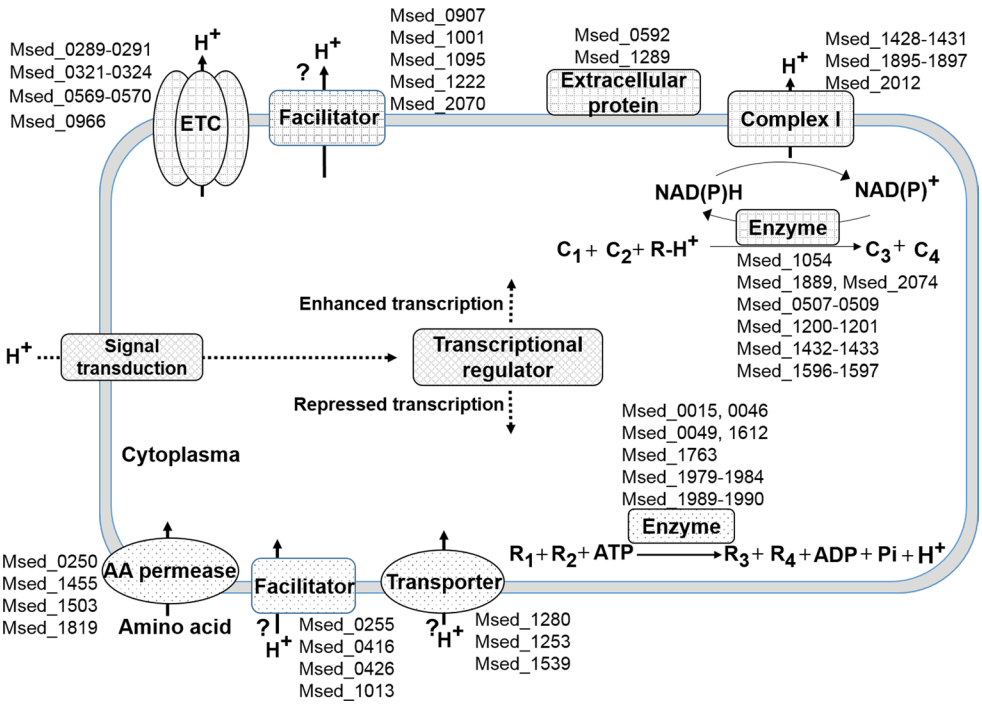

Fig. 5 A schematic model for the resistance of H^+ for *M. sedula* SARC-M 1. ETC: electron transfer chain; *C1, C2* the substrates of acid-consuming reaction, *C3, C4* the products of acid-consuming reaction, *R1, R2* the substrates of acid-generating reaction, *R3, R4* the products of acid-generating reaction

subunits of the bacterial formate hydrogenlyase complex that involved in the depletion of formate in the cytoplasm of *E. coli* were also upregulated [43]. The six genes (Msed_1979 to Msed_1984) encoding enzymes involved in purine biosynthesis and Msed_1763 that encodes an enzyme for pyrimidine metabolism were all only downregulated in pH 0.95 grown *M. sedula* SARC-M 1 not in the pH 1.50-grown wild type *M. sedula*. All of these catalyze acid-generating reactions. To summarize the overall effect of these reactions, a schematic model for acid resistance of *M. sedula* SARC-M 1 was proposed here based on the differential gene expression pattern under acid stress (Fig. 5). A high concentration of extracellular H^+ altered the expression pattern of transcriptional regulators that would trigger genome-wide transcriptional responses to acid stress. This would occur by; (1) enhancing expression of genes encoding outer membrane proteins, and membrane complexes/facilitators that extrude H^+, or enzymes that catalyzing H^+-consuming reaction, and (2) reducing the expression of genes encoding enzymes that catalyze H^+-generating reactions and transporters or amino permeases that promote the uptake of H^+.

Conclusion

This study reports the isolation and characterization of an acid-adapted derivative of *M. sedula* produced using adaptive evolution. *M. sedula* SARC-M1 exhibited more rapid heterotrophic growth and increased enargite bioleaching capacity as compared to its parental strain under extreme acidity. Mutations occurring in the genome of *M. sedula* SARC-M 1 and its altered transcriptome accounted for its acid resistance phenotype. The altered transcriptome was likely a stress response supported by mutation. The overall effect of the altered transcriptome was to enhance H^+ extrusion and to reduce both H^+ uptake and intracellular H^+ generation as an adaptive response to increased external acidity.

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