

Supplemental Materials

Ferric uptake regulator provides a new strategy for acidophile adaptation to acidic ecosystems

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Supplemental Materials and Methods

Plasmid construction, protein expression and purification

The *fur* gene was PCR-amplified from *A. caldus* MTH-04 chromosome and inserted into the *Nde* I and *Xho* I sites of pET22b and the resulting plasmid was transformed into *E. coli* DH5 α cells. To study AcFur protein properties, three AcFur mutants (Site I (SI) H31A/H88A, Site II (SII) E106A/H123A, SiteI and SiteII (SI&SII) H31A/H88A/E106A/H123A) were generated by site-directed mutagenesis. All plasmids were confirmed by DNA sequencing (GENEWIZ, Tianjin, China).

E. coli BL21 Star harboring the *fur* and *fur* mutants plasmids were grown in LB medium of 0.01% FeCl₂ containing 100 μ g/mL Amp until the OD₆₀₀ = 0.8 and were induced by adding isopropyl β -D-1-thiogalactopyranoside (IPTG) (0.2 mM, final concentration) and incubating overnight at 18 °C. The cells were harvested by centrifugation at 8000 \times g for 15 min at 4 °C and lysed by ultrasonication in lysis buffer (25 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.2 mM phenyl methane sulfonyl fluoride). After centrifugation at 17 000 \times g for 1 h at 4 °C, the supernatant was loaded to a Ni-NTA column (GE Healthcare), followed by a

24 washing buffer (25 mM Tris-HCl pH 8.0, 200 mM NaCl, 20 mM imidazole). The His-tagged AcFur and
25 AcFur mutants were eluted with elution buffer (25 mM Tris-HCl pH 8.0, 100 mM NaCl, and 250 mM
26 imidazole). Purified AcFur and AcFur mutants were analyzed by a 15% sodium dodecyl sulfate-
27 polyacrylamide gel electrophoresis (SDS-PAGE) followed by coomassie brilliant blue R-250 staining.

28 **Bioinformatics analysis of conservation of Fur proteins in the bacteria of AMD ecosystems**

29 The evolutionary conservation structure and sequences of Fur were assessed using the ConSurf
30 server(1). The ConSurf web servers calculated the evolutionary conservation of amino acid positions in the
31 AcFur using an empirical Bayesian paradigm. The conservation scores were grouped into a nine grades scale
32 and projected onto the structure and on the MSA for visualization.

33 **Supplemental Figures**

34 **Fig. S1 Construction procedure of Δfur and complementation strains.** (A) Schematic representation of
35 markerless gene knockout procedure. (B&C) Confirmation of Δfur by PCR. Positions of verification primers
36 used for PCR to confirm *fur* deletion. Lanes 1, 3, and 5, PCR amplifications from *A. caldus* MTH-04 wild
37 type chromosome with primers F1/R1, F2/R2 and F3/R3, respectively. Lanes 2, 4, and 6, PCR
38 amplifications from the *A. caldus* MTH-04 Δfur chromosome with primers F1/R1, F2/R2 and F3/R3,
39 respectively. (D&E) Confirmation of complementation strains by PCR. Positions of verification primers
40 used for PCR to confirm complementation mutagenesis. Lanes 1, 2, 3, 4, 5, and 6, PCR amplifications with
41 Δfur (*fur*), Δfur (S I), Δfur (S II), Δfur (S I & II), Δfur , and WT chromosome, respectively. UHA and DHA
42 represent upstream and downstream homologous arms, respectively.

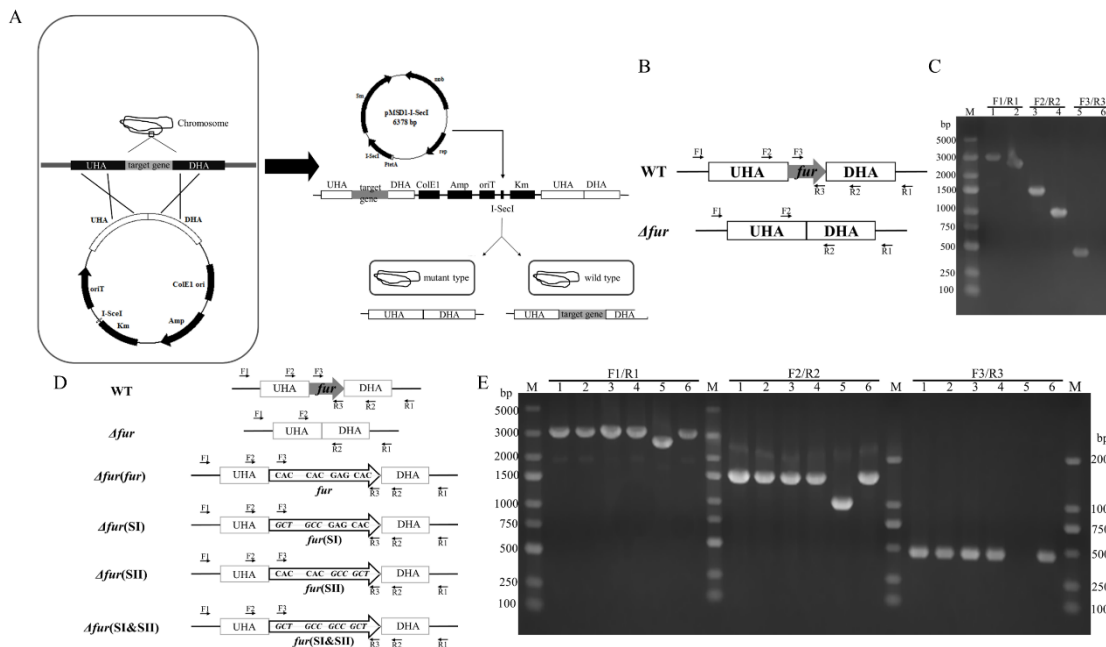


Fig. S2 Structure and sites conservation analysis of AcFur proteins and its homologs based on

Acidithiobacillus. (A&B) The nine-color conservation grades are projected onto the structure and sequence of AcFur protein. The variable positions (grade 1) colored turquoise, the intermediately conserved positions (grade 5) colored white, the most conserved positions (grade 9) colored maroon.

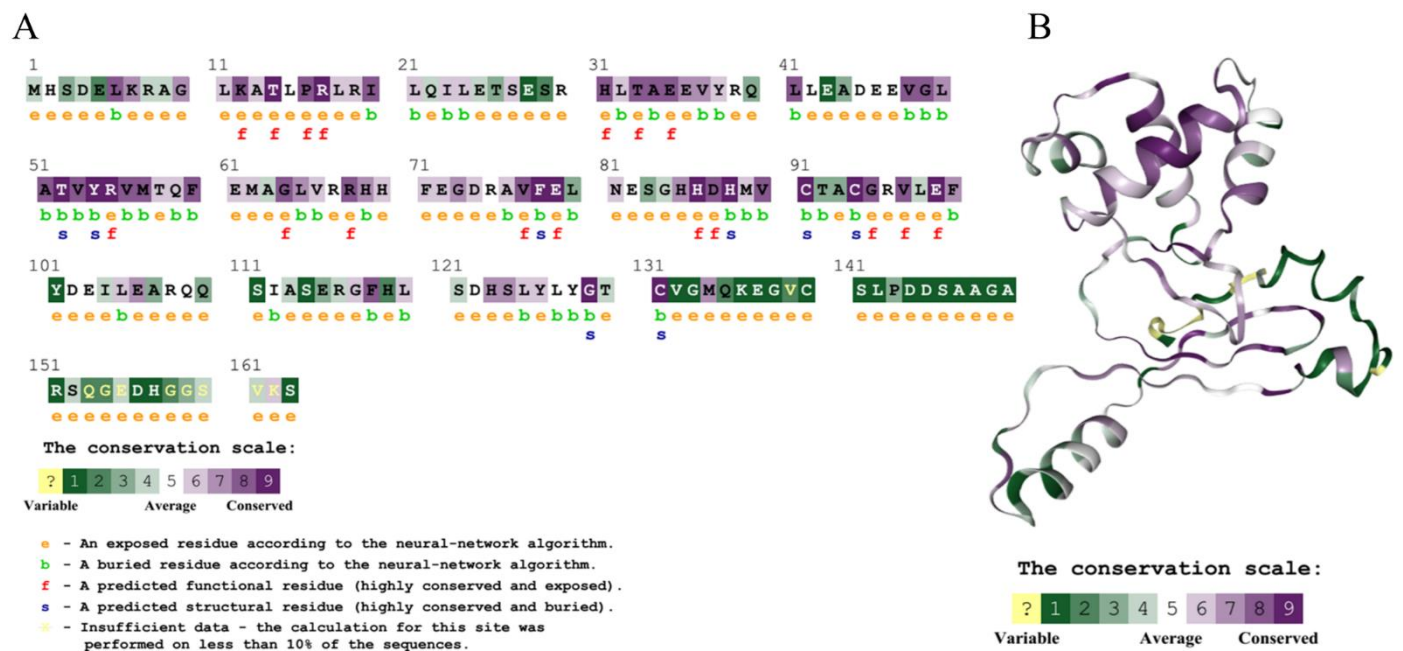
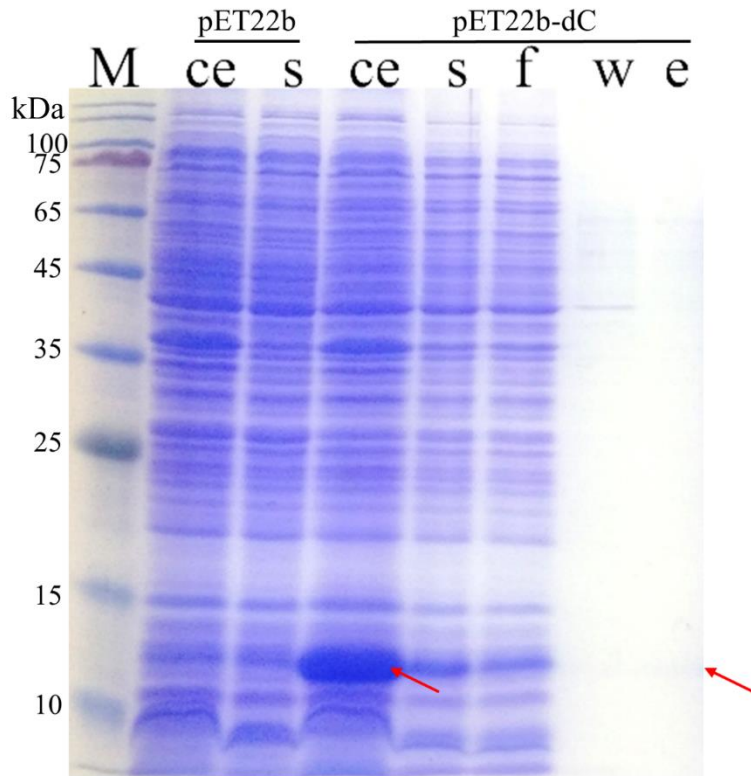


Fig. S3 Soluble test of Recombinant protein pET22b-dC. Recombinant protein pET22b-dC was pointed by red arrows. Lane M: M5 Prestained Protein Ladder Maker; lanes ce-s corresponding to the total proteins and supernatant of pET-22b; lanes ce-e corresponding to the crude extraction (ce), supernatant (s), flow (f),

52 washing (w), elution (e) of pET-22b-dC.



53

54 **Supplemental Table**

55 **Table S1 Occurrence of fur and DtxR genes in published genomic information of AMD bacteria.**

Phylum	Bacteria	Assembly level	Fur	DtxR	
<i>α</i> Proteobacteria	<i>Acidiphilium cryptum</i> JF-5	complete genome	NO	NO	
	<i>Acidiphilium multivorum</i> AIU301	complete genome	NO	NO	
	<i>Acidiphilium angustum</i> ATCC 35903	Scaffold	NO	NO	
	<i>Acidiphilium rubrum</i> ATCC 35905	Scaffold	NO	NO	
	<i>Acidocella aminolytica</i> 101	Scaffold	WP_048880410.1	NO	
	<i>Acidocella facilis</i> ATCC 35904	Contig	WP_026439175.1	NO	
	<i>Acidomonas methanolica</i> NBRC 104435	Scaffold	WP_081797598.1	NO	
	<i>β</i> Proteobacteria	<i>Ferroplasma myxofaciens</i> P3G	Contig	WP_031595537.1	NO
		<i>Thiomonas intermedia</i> K12	complete genome	WP_013121860.1	NO
		<i>Thiomonas arsenitoxydans</i> 3As	Chromosome	WP_013104191.1	NO
<i>Thiomonas delicata</i> DSM 16361		Scaffold	WP_094159584.1	NO	
<i>Thiomonas bhubaneswarensis</i> DSM 18181		Scaffold	WP_055449419.1	NO	

<i>γProteobacteria</i>	<i>Acidiferrobacter thiooxydans</i>			
	ZJ	Contig	WP_083996317.1	NO
	<i>Acidiferrobacter sp. SPIII_3</i>	complete genome	WP_110138519.1	NO
	<i>Acidihalobacter ferrooxidans</i>			
	V8	Chromosome	WP_076835706.1	NO
	<i>Acidihalobacter prosperus</i>			
<i>Acidithiobacilla</i>	F5	complete genome	WP_070077907.1	NO
	<i>Metallibacterium scheffleri</i>			
	DKE6	Contig	WP_081127454.1	NO
	<i>Acidithiobacillus thiooxidans</i>			
	ATCC 19377	Scaffold	WP_010641352.1	NO
	<i>Acidithiobacillus ferrooxidans</i>			
	ATCC 23270	complete genome	WP_012536062.1	NO
	<i>Acidithiobacillus caldus</i>	complete genome	WP_004869019.1	NO
	<i>Acidithiobacillus ferrivorans</i>			
	SS3	complete genome	WP_014030069.1	NO
	<i>Acidithiobacillus ferridurans</i>			
	JCM 18981	complete genome	WP_113526343.1	NO
<i>Acidithiobacillus albertensis</i>				
DSM 14366	Scaffold	WP_010641352.1	NO	
<i>δproteobacteria</i>	<i>Desulfomonile tiedjei</i>			
	DSM 6799	complete genome	WP_014810043.1	WP_014809611.1
<i>Acidobacteria</i>	<i>Acidobacterium ailaoui</i>			
	PMMR2	Contig	WP_026442842.1	WP_049961364.1
	<i>Acidobacterium capsulatum</i>			
	ATCC 51196	complete genome	WP_015898090.1	ACO33208.1
	<i>Granulicella mallensis</i>			
	MP5ACTX8	complete genome	WP_014263331.1	AEU38646.1
	<i>Granulicella pectinivorans</i>			
	DSM 21001	Contig	WP_089840414.1	WP_089837736.1
	<i>Granulicella rosea</i>			
	DSM 18704	Contig	WP_089407384.1	WP_142988166.1
	<i>Granulicella tundricola</i>			
	MP5ACTX9	complete genome	WP_013572743.1	ADW70438.1
<i>Terriglobus saanensis</i>				
SP1PR4	complete genome	ADV82922.1	ADV82279.1	
<i>Terriglobus roseus</i>				
DSM 18391	complete genome	NO	AFL86563.1	
<i>Nitrospirae</i>	<i>Leptospirillum rubarum</i>	Scaffold	WP_014959897.1	NO
	<i>Leptospirillum ferriphilum</i>			
	ML-04/YSK	complete genome	WP_014959897.1	NO
	<i>Leptospirillum ferrooxidans</i>			
	C2-3	complete genome	WP_014448348.1	NO
	<i>Leptospirillum sp. Group II 'CF-1'</i>	complete genome	AKS24796.1	NO
	<i>Leptospirillum sp. Group II 'C75'</i>	Scaffold	EIJ75953.1	NO

	<i>Leptospirillum ferro Diazotrophum</i>	/	EES52611.1	NO
	<i>Leptospirillum sp. Group IV 'UBA BS'</i>	Scaffold	NO	NO
<i>Actinobacteria</i>	<i>Acidimicrobium ferrooxidans</i> DSM 10331	complete genome	ACU53263.1	NO
	<i>Acidithrix ferrooxidans</i> Py-F3	Contig	WP_052604974.1	NO
	<i>Candidatus Koribacter versatilis</i> Ellin345	complete genome	ABF42368.1	NO
	<i>Candidatus Solibacter usitatus</i> Ellin6076	complete genome	ABJ82847.1	ABJ83168.1
	<i>Ferrimicrobium acidiphilum</i> DSM 19497	Scaffold	KJE75465.1	NO
	<i>Ferrithrix thermotolerans</i> DSM 19514	Scaffold	SHE49700.1	NO
<i>Firmicutes</i>	<i>Sulfobacillus benefaciens</i>	Scaffold	PSR32246.1	NO
	<i>Sulfobacillus acidophilus</i> DSM 10332	complete genome	AEJ38577.1	NO
	<i>Sulfobacillus thermosulfidooxidans</i> DSM 9293	Scaffold	WP_051351132.1	NO
	<i>Sulfobacillus sp.</i> Hq2	Scaffold	WP_103375064.1	NO
	<i>Sulfobacillus thermotolerans</i> Kr	/	AUW95357.1	NO
	<i>Alicyclobacillus acidiphilus</i> NBRC 100859	Contig	WP_067621411.1	WP_067621532.1
	<i>Alicyclobacillus acidocaldarius</i> DSM 446	complete genome	ACV59616.1	WP_012811361.1
	<i>Alicyclobacillus acidoterrestris</i> ATCC 49025	Contig	EPZ52565.1	EPZ52800.1
	<i>Alicyclobacillus macrosporangiidus</i> DSM 17980	Scaffold	WP_074951538.1	WP_074955662.1
	<i>Alicyclobacillus mali</i> NBRC 102425	Contig	WP_067846234.1	WP_067845958.1
	<i>Alicyclobacillus herbarius</i> DSM 13609	Scaffold	WP_026961358.1	WP_026960854.1
	<i>Alicyclobacillus contaminans</i> DSM 17975	Scaffold	WP_026975363.1	WP_035485231.1
	<i>Alicyclobacillus hesperidum</i>	Scaffold	NO	WP_006446406.1
	<i>Alicyclobacillus montanus</i> USBA-GBX-503	Scaffold	WP_072873733.1	WP_072872655.1
	<i>Alicyclobacillus ferrooxydans</i> TC-34	Contig	WP_054968265.1	WP_054970710.1
	<i>Alicyclobacillus shizuokensis</i> NBRC 103103	Contig	WP_067927130.1	WP_067923196.1

	<i>Alicyclobacillus sendaiensis</i> NBRC 100866	Contig	WP_062305267.1	WP_062307455.1
	<i>Alicyclobacillus</i> <i>kakegawensis</i> NBRC 103104	Contig	WP_067932739.1	WP_067930834.1
	<i>Alicyclobacillus</i> <i>tengchongensis</i>	Contig	NO	WP_006446406.1
	<i>Alicyclobacillus sacchari</i> DSM 17974	Scaffold	WP_134158132.1	WP_134158038.1
	<i>Alicyclobacillus pomorum</i> DSM 14955	Scaffold	WP_026964647.1	NO
	<i>Alicyclobacillus vulcanalis</i> DSM 16176	Scaffold	WP_143232488.1	WP_076348602.1
	<i>Desulfosporosinus</i> <i>acidiphilus</i> SJ4	complete genome	AFM41980.1	NO
	<i>Desulfosporosinus youngiae</i> DSM 17734	Chromosome	EHQ90093.1	NO
	<i>Desulfosporosinus orientis</i> DSM 765	complete genome	AET68842.1	NO
	<i>Desulfosporosinus lacus</i> DSM 15449	Scaffold	SHH87716.1	SHI82465.1
	<i>Desulfosporosinus hippei</i> DSM 8344	Scaffold	SDG84826.1	NO
	<i>Desulfosporosinus meridiei</i> DSM 13257	complete genome	AFQ44521.1	NO
	<i>Desulfosporosinus</i> <i>fructosivorans</i> 63.6F	Scaffold	WP_135548847.1	WP_135550222.1
<i>Aquificae</i>	<i>Hydrogenobaculum</i> sp.	complete genome	WP_012513885.1	NO
<i>Euryarchaeota</i>	<i>Thermoplasma acidophilum</i> DSM 1728	complete genome	NO	DtxR 406J_A
	<i>Thermoplasma volcanium</i> GSS1	complete genome	BAB59437.1	BAB60108.1
	<i>Thermoplasma</i> sp. Kam2015	Contig	WP_110642587.1	WP_110641559.1
	<i>Cuniculiplasma divulgatum</i> PM4	complete genome	WP_021789929.1	NO
	<i>Picrophilus oshimae</i> DSM 9789	Contig	NO	SMD31358.1
	<i>Picrophilus torridus</i> DSM 9790	complete genome	NO	AAT43733.1
	<i>Acidiplasma aeolicum</i>	Contig	NO	WP_048101435.1
	<i>Acidiplasma cupricumulans</i>	Contig	NO	WP_048101435.1
	<i>Acidiplasma</i> sp. MBA-1	Contig	NO	WP_048101435.1
	<i>Ferroplasma acidarmanus</i> fer1	complete genome	NO	AGO60290.1
	<i>Ferroplasma acidiphilum</i>	complete genome	NO	WP_081142639.1
	<i>Ferroplasma thermophilum</i>	/	NO	NO
	<i>Ferroplasma</i> <i>cyprexacervatum</i>	Contig	NO	WP_048101435.1

<i>Crenarchaeota</i>	<i>Ferroplasma cupricumulans</i>	Contig	NO	WP_048101435.1
	<i>Acidianus hospitalis</i> W1	complete genome	NO	AEE93435.1
	<i>Acidianus sulfidivorans</i> JP7	complete genome	NO	AWR97065.1
	<i>Acidianus brierleyi</i> DSM 1651	complete genome	NO	WP_110270607.1
	<i>Acidianus manzaensis</i> YN-25	complete genome	NO	ARM75708.1
	<i>Candidatus Acidianus copahuensis</i>	Contig	NO	WP_048100246.1
	<i>Acidilobus saccharovorans</i> 345-15	complete genome	NO	NO
	<i>Acidilobus</i> sp. 7A	complete genome	NO	NO
	<i>Metallosphaera sedula</i>	complete genome	NO	WP_012021943.1
	<i>Metallosphaera hakonensis</i> JCM 8857	complete genome	NO	AWR99707.1
	<i>Metallosphaera yellowstonensis</i>	complete genome	NO	WP_048088038.1
	<i>Metallosphaera cuprina</i> Ar-4	Scaffold	NO	AEB94380.1
	<i>Metallosphaera prunae</i>	complete genome	NO	QCO30441.1
	<i>Sulfolobus islandicus</i>	Complete	NO	WP_012711465.1
	<i>Sulfolobus acidocaldarius</i>	Complete	NO	WP_011277038.1
	<i>Sulfolobus metallicus</i>	Contig	NO	WP_054838202.1
	<i>Sulfurisphaera tokodaii</i>	complete genome	NO	WP_010980111.1
	<i>Saccharolobus solfataricus</i> P2	complete genome	NO	AAK40972.1

56 **References**

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