

## Supplementary Materials

# Expression, Purification and Characterization of a Novel Rusticyanin from the Psychrotolerant *Acidithiobacillus ferrivorans*

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Text S1. The Specific primers used as a template for PCR amplification of the sequence area containing the type-C rusticyanin gene

The Specific primers used as a template for polymerase chain reaction (PCR) for amplification of the sequence area containing the type-C rusticyanin gene were designed according to the whole genome sequence of *A. ferrivorans* SS3 using the Primer Premier 5.0 software.

The forward primer was 5' -CCAATCCGGCATGAGACTTC-3' , start from -58 to -38 site in the upstream of 5' terminal of the *rus* gene at locus 0470.

The reverse primer was 5' - ACTTCCGTTTCAACCATGGC-3' , start from 147 to 127 site in the downstream of 3' terminal of the *rus* gene at locus 0470.

Table S1. The different residues and their property differences for the type-C rusticyanin from *A. ferrivorans* SS3 comparing to the type-A from *A. ferrooxidans* ATCC 23270 and the type-B from *A. ferrooxidans* JCM 3865, respectively. The different amino acid residues are identified from the sequence alignment of rusticyanins shown as Figure S1. The amino acid residue positions are determined according the built molecular structure of the type-C rusticyanin from *A. ferrivorans* SS3 shown as Figure 6a. The hydrophobicity differences between amino acid residues are computed based on the hydrophobicity values in the Kyte and Doolittle hydrophobicity values [31]. The net charge differences at pH 2.0 between amino acid residues are computed according to the pKa values in the initial pKa values for standard amino acids [32].

Type-C rusticyanin from <i>A. ferrivorans</i> SS3		Type-A rusticyanin from <i>A. ferrooxidans</i> ATCC 23270			Type-B rusticyanin from <i>A. ferrooxidans</i> JCM 3865		
Amino acid	Position	Amino acid	Hydrophobicity difference	Charge difference at pH 2.0	Amino acid	Hydrophobicity difference	Charge difference at pH 2.0
<b>Thr1</b>	surface	Gly1	-0.3	0	Ala1	-2.5	0
Pro2	surface	Thr2	-0.9	0			
Ser6	surface	Thr6	-0.1	0			
<b>Ser9</b>	surface	Glu9	2.7	0	Met9	-2.7	0
Leu18	surface	Met18	1.9	0			
<b>Val20</b>	surface	Glu20	7.7	0	Ala20	2.4	0
Ser23	surface	Thr23	-0.1	0			
<b>Glu25</b>	surface	Lys25	0.4	-1	Lys25	0.4	-1
Lys29	surface	Asp29	-0.4	1			
Ile56	buried	Val56	0.3	0			
Val59	surface	Lys59	8.1	-1			
Asp65	surface	Glu65	0	0			
Ile74	buried	Val74	0.3	0			
Phe87	buried				Leu87	-1	0
<b>Asn101</b>	surface	Val101	-7.7	0	Val101	-7.7	0
<b>Lys103</b>	surface	Asp103	-0.4	1	Asn103	-0.4	1
Val106	surface				Ile106	-0.3	0
Asp117	surface				Ser117	-2.7	0
Lys119	surface				Gly119	-3.5	1
His128	surface				Arg128	1.3	0
Thr130	surface				Ala130	-2.5	0
<b>Total difference</b>		<b>15</b>	<b>11.5</b>	<b>0</b>	<b>12</b>	<b>-19.2</b>	<b>1</b>

Table S2. Gene contexts of the rusticyanin genes in *A. ferrivorans* SS3 [17] and *A. ferrooxidans*. All involved sequences are from the public sequence database of NCBI (National Center for Biotechnology Information) ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov) (accessed on 30 June 2023)). The gene background information of *rus* genes from *A. ferrooxidans* SS3 was from the whole genome sequence of NCBI accession NC\_015942. The gene background information of the *rusA* from *A. ferrooxidans* ATCC 23270 is from the whole genome sequence of NCBI accession NC\_011761. The gene background information of the *rusB* from *A. ferrooxidans* 3865 was from the sequence of NCBI accession AB094633 and reported by Kazuhiro Sasaki et.al [13,33].

locus	name	length	annotation
<i>rus</i> operon from <i>A. ferrivorans</i> SS3			
1872	<i>cyc2</i>	1458	cytochrome c
1871	<i>cyc1</i>	690	cytochrome c class I
1870	<i>orf</i>	552	hypothetical cupredoxin family protein
1869	<i>coxB</i>	765	cytochrome c oxidase subunit II
1868	<i>coxA</i>	1884	cytochrome c oxidase subunit I
1867	<i>coxC</i>	555	cytochrome c oxidase, aa3-type subunit III
1866	<i>coxD</i>	195	cytochrome c oxidase, aa3-type subunit IV
1865		141	hypothetical protein
<b>1864</b>	<b><i>rusA</i></b>	<b>564</b>	<b>type-A rusticyanin</b>
1863		954	hypothetical protein
<i>A. ferrivorans</i> SS3			
1717		480	hypothetical protein
<b>1718</b>	<b><i>rusB</i></b>	<b>570</b>	<b>type-B Rusticyanin</b>
1719		333	hypothetical protein
1720	<i>dinB</i>	1200	DNA polymerase IV
1721	<i>abrB</i>	240	SpoVT/AbrB like domain-containing protein
1722	<i>pilT</i>	411	PilT protein domain-containing protein
<i>A. ferrivorans</i> SS3			
0467	<i>phd</i>	243	prevent-host-death family protein
0468	<i>pilT</i>	396	PilT protein domain-containing protein
0469	<i>cusF</i>	345	heavy metal efflux system protein, copper binding periplasmic protein
<b>0470</b>	<b><i>rusC</i></b>	<b>570</b>	<b>type-C rusticyanin</b>
0471	<i>orf1</i>	300	hypothetical protein
0472	<i>orf2</i>	408	hypothetical protein
<i>rus</i> operon from <i>A. ferrooxidans</i> ATCC 23270			
3153	<i>cyc2</i>	1458	cytochrome c
3152	<i>cyc1</i>	693	cytochrome c class I
3151	<i>orf</i>	552	hypothetical cupredoxin family protein
3150	<i>coxB</i>	765	cytochrome c oxidase subunit II
3149	<i>coxA</i>	1884	cytochrome c oxidase subunit I
3148	<i>coxC</i>	555	cytochrome c oxidase, aa3-type subunit III
3147	<i>coxD</i>	195	cytochrome c oxidase, aa3-type subunit IV
<b>3146</b>	<b><i>rusA</i></b>	<b>564</b>	<b>type-A rusticyanin</b>

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*A. ferrooxidans JCM 3865*

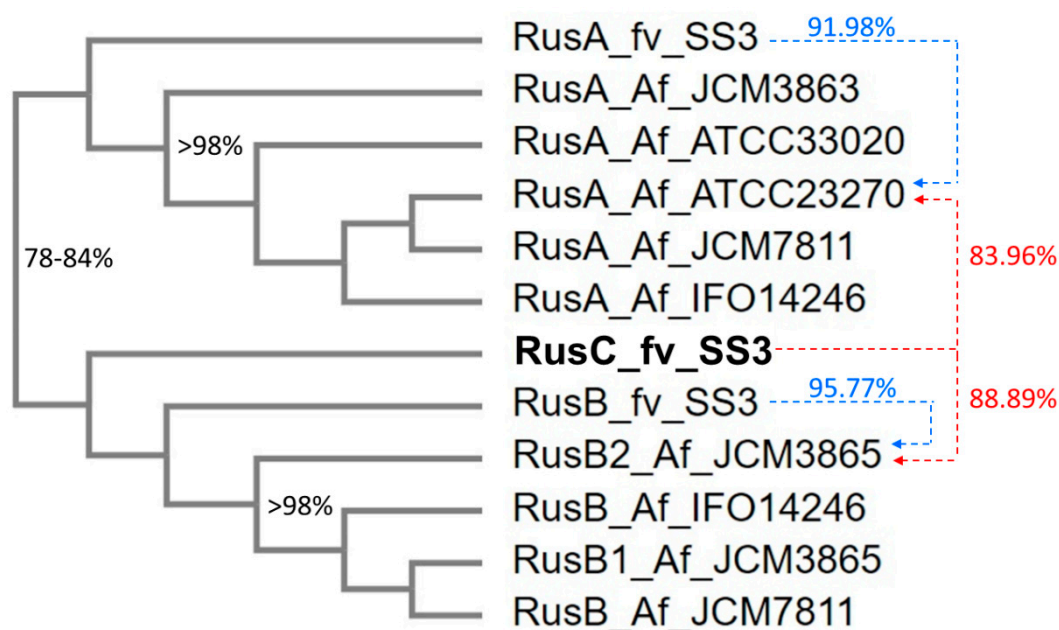
***orf*** hypothetical protein

***rusB*** 570 **type-B rusticyanin**

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	└-----SignalP (34)-----┐ └-----Lateral Belt (35)-----┘	
RusC_fv_SS3	MNTQNKVMTNNGRYYAAGLSAILAATLGITTAMATPLDTSWKSATLPQVKALLVKDSGEVSGK	63
RusB2_Af_JCM3865	MNTQIKPTMHKGRHIAAGLSTILAAALGISTAMAAPLDTSWKMATLPQVKALLAKDSGKVS	63
RusB_Af_IF014246	-----HKGRHVAAGLSTILAAALGISTAMAAPLDTSWKMATLPQVKALLAKDSGKVS	54
RusB1_Af_JCM3865	-----HKGRHVAAGLSTILAAALGISTAMAAPLDTSWKMATLPQVKALLAKDSGKVS	54
RusB_Af_JCM7811	-----PTMHKGRHVAAGLSTILAAALGISTAMAAPLDTSWKMATLPQVKALLAKDSGKVS	57
RusB_fv_SS3	MNTQIKPTMHKGRYIAAGLSTVLAAALGMSTAMAAPLDTSWKMATLPQVKALLAKDSGTVSGK	63
RusA_fv_SS3	MNT--QTKMQKNWYVSVGAAAVLAATVGMGTAMAGTLDSSWKEATLPQVKAMLQKDTGKVS	61
RusA_Af_JCM3863	-----KKNWYVTVGAAAAAATVGMGTAMAGTLDTTWKEATLPQVKAMLEKDTGKVS	54
RusA_Af_ATCC33020	--MYTQNTMKKNWYVTVGAAAAAATVGMGTAMAGTLDSTWKEATLPQVKAMLEKDTGKVS	61
RusA_Af_ATCC23270	--MYTQNTMKKNWYVTVGAAAAAATVGMGTAMAGTLDTTWKEATLPQVKAMLEKDTGKVS	61
RusA_Af_JCM7811	--MYTQNTMKKNWYVTVGAAAAAATVGMGTAMAGTLDTTWKEATLPQVKAMLEKDTGKVS	61
RusA_Af_IF014246	--MYTQNTMKKNWYVTVGAAAAAATVGMGTAMAGTLDTTWKEATLPQVKAMLEKDTGKVS	61
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RusC_fv_SS3	TVTYSGKTVHVVAAAVLPGFPFSPFEIHDVKNPTLDIPAGATVDITFINTNKGFCHSFDITKK	126
RusB2_Af_JCM3865	TVTYSGKTVHVVAAAVLPGFPFSPFEIHDVKNPTLDIPAGATVDITFINTNKGFCHSLDITKK	126
RusB_Af_IF014246	TVTYSGKTVHVVAAAVLPGFPFSPFGIHDVKNPTLDIPAGATVDITFINTNKGFCHSLDITKK	117
RusB1_Af_JCM3865	TVTYSGKTVHVVAAAVLPGFPFSPFEIHDVKNPTLDIPAGANVDITFINTNKGFCHSLDITKK	117
RusB_Af_JCM7811	TVTYSGKTVHVVAAAVLPGFPFSPFEIHDVKNPTLDIPAGATVDITFINTNKGFCHSLDITKK	120
RusB_fv_SS3	TVTYSGKTVHVVAAAVLPGFPFSPFEIHDVKNPTLEIPAGATVDITFINTNKGFCHSLDITKK	126
RusA_fv_SS3	TVTYSGKTVHVVAAAVLPGFPFSPFEVHDKKNPTLDIPAGATVDVTFINTNKGFCHSFDITQK	124
RusA_Af_JCM3863	TVTYSGKTVHVVAAAVLPGFPFSPFEVHDKKNPTLEIPRGATVDVTFINTNKGFCHSFDITKK	117
RusA_Af_ATCC33020	TVTYSGKTVHVVAAAVLPGFPFSPFEVHDKKNPTLEIPAGATVDVTFINTNKGFCHSFDITKK	124
RusA_Af_ATCC23270	TVTYSGKTVHVVAAAVLPGFPFSPFEVHDKKNPTLEIPAGATVDVTFINTNKGFCHSFDITKK	124
RusA_Af_JCM7811	TVTYSGKTVHVVAAAVLPGFPFSPFEVHDKKNPTLEIPAGATVDVTFINTNKGFCHSFDITKK	124
RusA_Af_IF014246	TVTYSGKTVHVVAAAVLPGFPFSPFEVHDKKNPTLEIPAGATVDVTFINTNKGFCHSFDITKK	124
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RusC_fv_SS3	GPPYAVMPNIIKPIVAGTGFSPVPKDGKFGYTDFTWHPTAGTYYYYVCQIPGHAATGMFGKIVVK	189
RusB2_Af_JCM3865	GPPYAVMPVINPIIAGTGFSPVPKSGGFGYTDFTWRPAAGTYYYYVCQIPGHAATGMFGKIVVK	189
RusB_Af_IF014246	GPPYAVMPVIDPIIAGTGFSPVPKSGGFGYTDFTWRPAAGTYYYYVCQIPGHAATGMFG-----	175
RusB1_Af_JCM3865	GPPYAVMPVIDPIIAGTGFSPVPKSGGFGYTDFTWRPAAGTYYYYVCQIPGHAATGMFG-----	175
RusB_Af_JCM7811	GPPYAVMPVIDPIIAGTGFSPVPKSGGFGYTDFTWRPAAGTYYYYVCQIPGHAATGMFG-----	178
RusB_fv_SS3	GPPYAVMPAINPIIAGTGFSPVPKSGGFGYTDFTWHPTAGTYYYYVCQIPGHAATGMFGKIVVK	189
RusA_fv_SS3	TPPFAVMPVIDPIVAGTGFSPVPKDGKFGYTNTFWHPTAGTYYYYVCQIPGHAATGMFGKIIVK	187
RusA_Af_JCM3863	GPPYAVMPVIDPIVAGTGFSPVPKDGKFGYTDFTWHPTAGTYYYYVCQIPGHAATGMFG-----	175
RusA_Af_ATCC33020	GPPYAVMPVIDPIVAGTGFSPVPKDGKFGYTDFTWHPTAGTYYYYVCQIPGHAATGMFGKIIVK	187
RusA_Af_ATCC23270	GPPYAVMPVIDPIVAGTGFSPVPKDGKFGYTDFTWHPTAGTYYYYVCQIPGHAATGMFGKIVVK	187
RusA_Af_JCM7811	GPPYAVMPVIDPIVAGTGFSPVPKDGKFGYTDFTWHPTAGTYYYYVCQIPGHAATGMFG-----	182
RusA_Af_IF014246	GPPYAVMPVIDPIVAGTGFSPVPKDGKFGYTDFTWHPTAGTYYYYVCQIPGHAATGMFG-----	182
	***:**** *.*:*****.* *****:***:.*:*****:**:**:**:***	

**Figure S1.** Sequence alignment of rusticyanins from *A. ferrivorans* SS3 and various *A. ferrooxidans* sources. RusC\_fv\_SS3: the type-C rusticyanin from *A. ferrivorans* SS3, locus at 0470; RusB2\_Af\_JCM3865: the type-B rusticyanin from *A. ferrooxidans* JCM 3865, Genebank accession number AB094633; RusB\_Af\_IFO14246: the type-B rusticyanin from *A. ferrooxidans* IFO 14246, Genebank accession number AB094636; RusB1\_Af\_JCM3865: the type-B rusticyanin from *A. ferrooxidans* JCM 3865, Genebank accession number AB094632; RusB\_Af\_JCM7811: the type-B Rusticyanin from *A. ferrooxidans* JCM 7811, Genebank accession number AB094635; RusB\_fv\_SS3: the type-B rusticyanin from *A. ferrivorans* SS3, locus at 1718; RusA\_fv\_SS3: the type-C rusticyanin from *A. ferrivorans* SS3, locus at 1864; RusA\_Af\_JCM3863: the type-A Rusticyanin from *A. ferrooxidans* JCM 3863, Genebank accession number AB094631; RusA\_Af\_ATCC33020: the type-A rusticyanin from *A. ferrooxidans* ATCC 33020, Genebank accession number AJ006456; RusA\_Af\_ATCC23270: the type-A rusticyanin from *A. ferrooxidans* ATCC 23270, locus at 3146 or PDB number 1CUR; RusA\_Af\_JCM7811: the type-A Rusticyanin from *A. ferrooxidans* JCM 7811, Genebank accession number AB094634; RusA\_Af\_IFO14246: the type-A rusticyanin from *A. ferrooxidans* IFO 14246, Genebank accession number AB094637;. The signal peptide sequences are in blue. The conserved residues ligated the copper factor are in red. The conserved cysteine residues are marked with \*, residues not conserved in all sequences but conserved in some sequences are marked with : or . based on the degree of conservation.



**Figure S2.** Phylogenetic tree of the rusticyanins from *A. ferrivorans* SS3 and various *A. ferrooxidans* sources according to Figure S1.