Supplementary Information

Culturing the ubiquitous freshwater actinobacterial acI lineage by supplying a biochemical 'helper' catalase

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Supplementary Text

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Description of two proposed '*Candidatus*' species

The average nucleotide identity (ANI) value calculated from genome sequences between strain IMCC25003 and '*Ca*. Planktophila sulfonica' MMS-IA-56 was 84% and between strains MCC26103 and '*Ca*. Planktophila lacus' MMS-21-148 was 78%, which were both below the 95~96% cut-off value for bacterial species demarcation[1,2]. The analysis of genomic DNA-DNA relatedness and differential phenotypic characteristics indicated that strains IMCC25003 and IMCC26103 each represent novel species of the genus '*Candidatus* Planktophila'. Because the two strains, however, did not grow on a defined medium or a synthetic medium but replicated only in complex natural lake water media, hampering the deposition of the acI strains in culture collections, we propose the provisional names '*Candidatus* Planktophila rubra' for strain IMCC25003 and '*Candidatus* Planktophila aquatilis' for strain IMCC26103.

'*Candidatus* Planktophila rubra' (ru'bra. L. fem. adj. *rubra* reddish, pertaining to the reddish color of cells)

Represented by a cultured bacterial strain, IMCC25003. Gram-positive, aerobic, red-pigmented, non-motile, and chemoheterotrophic. Cells are curved rods with biovolume of 0.041 μm^3, 0.46–1.23 μm (average 0.68 μm) long and 0.25–0.37 μm (average 0.30 μm) wide. Grows in FAMV+CM+AA supplemented with >0.5 U ml^{-1} catalase but does not grow in any liquid medium devoid of catalase and on any solid agar medium. Growth occurs at 10–30°C (optimum, 25°C). No single carbon sources enhance the cellular growth. Requires sulfur-containing amino acids (methionine and cysteine) but prefers methionine. The major fatty acids (>10%) are summed feature 3 ($C_{16:1}$ $\omega 7c$ and/or $C_{16:1}$ $\omega 7c$, 45.8%), $C_{16:0}$ (23.1%), and $C_{14:0}$ (18.2%). Strain IMCC25003 has a genome size of 1.354 Mbp with DNA G+C content of 49.1%. The complete genome sequence of strain IMCC25003 is available on GenBank (CP029557). Phylogenetically belongs to the acI-A1 tribe.

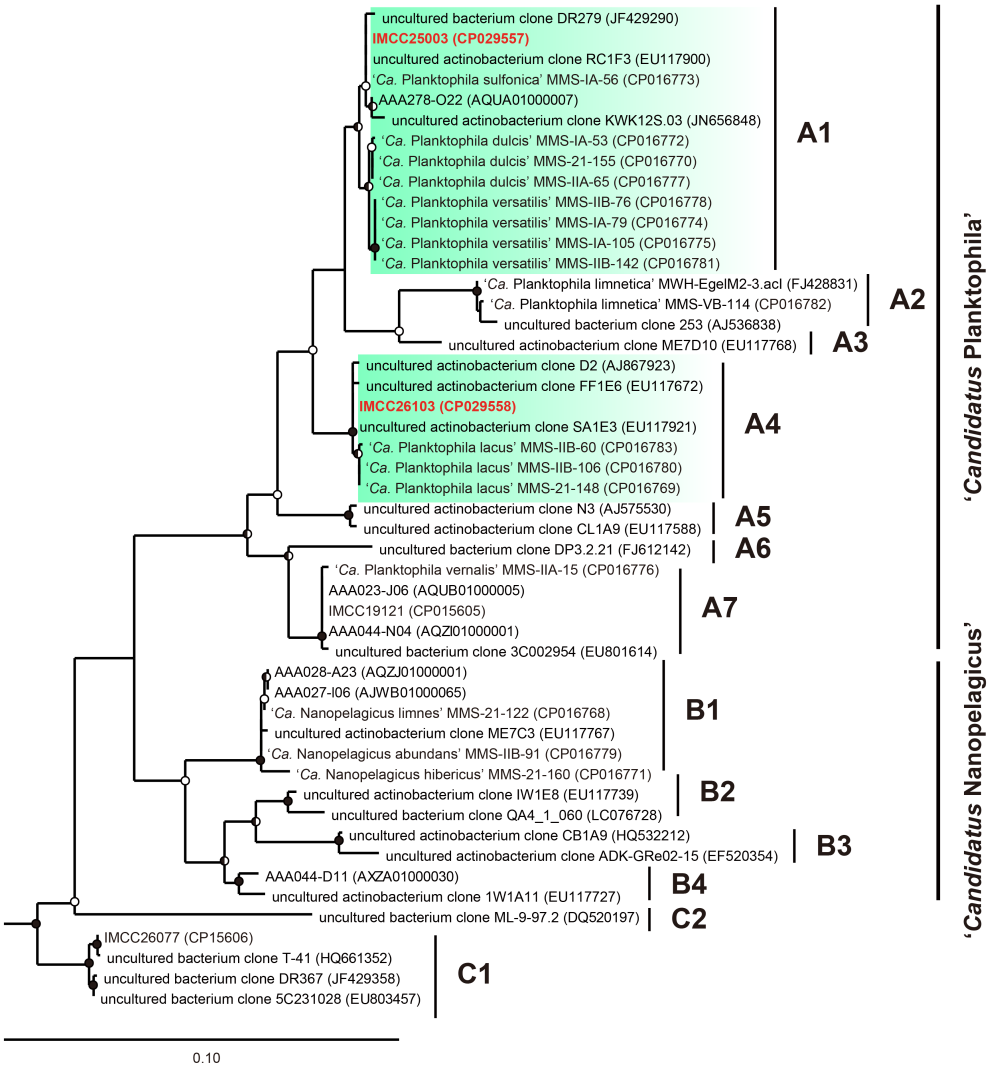
The representative strain IMCC25003 was isolated from a freshwater lake, Lake Soyang, Republic of Korea, using a dilution-to-extinction culturing.

'*Candidatus* Planktophila aquatilis' (a.qua.ti′lis. L. fem. adj. *aquatilis* living, growing, or found, in or near water, aquatic).

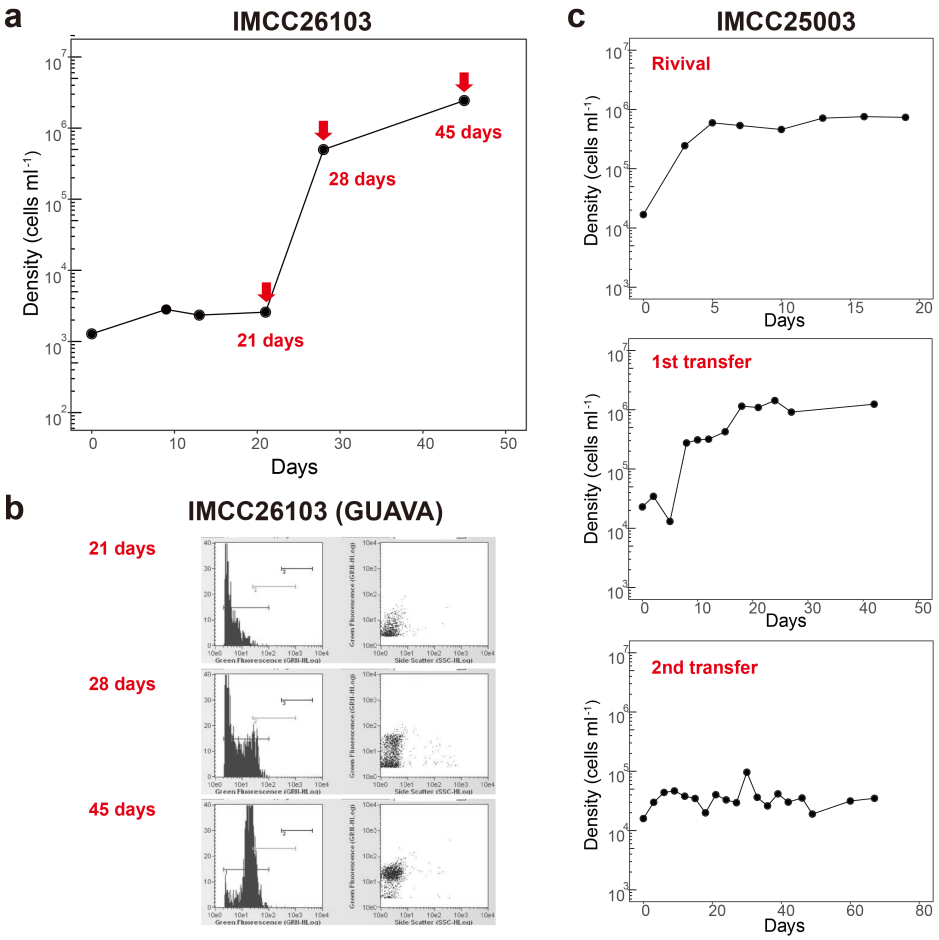
Represented by a cultured bacterial strain, IMCC26103. Gram-positive, aerobic, red-pigmented, non-motile, and chemoheterotrophic. Cells are curved rods with biovolume of 0.061 μm^3, 0.49–1.23 μm (average 0.88 μm) long and 0.22–0.39 μm (average 0.31 μm) wide. Grows in FAMV+CM+AA supplemented with >0.5 U ml^{-1} catalase but does not grow in any liquid medium devoid of catalase and on any solid agar medium. Growth occurs at 10–30°C (optimum, 25°C). D-ribose and D-glucose enhance the cellular growth. Requires sulfur-containing amino acids (methionine and cysteine) but prefers cysteine. The major fatty acids (>10%) are $C_{16:0}$ (28.5%), $C_{18:1}$ $\omega 9c$ (25.8%), summed feature 3 ($C_{16:1}$ $\omega 7c$ and/or $C_{16:1}$ $\omega 7c$, 12.3%), and $C_{18:0}$ (10.5%). Strain IMCC26103 has a genome size of 1.457 Mbp with DNA G+C content of 47.0%. The complete genome sequence of strain IMCC26103 is available on GenBank (CP029558). Phylogenetically belongs to the acI-A4 tribe.

The representative strain IMCC26103 was isolated from a freshwater lake, Lake Soyang, Republic of Korea, using a dilution-to-extinction culturing.

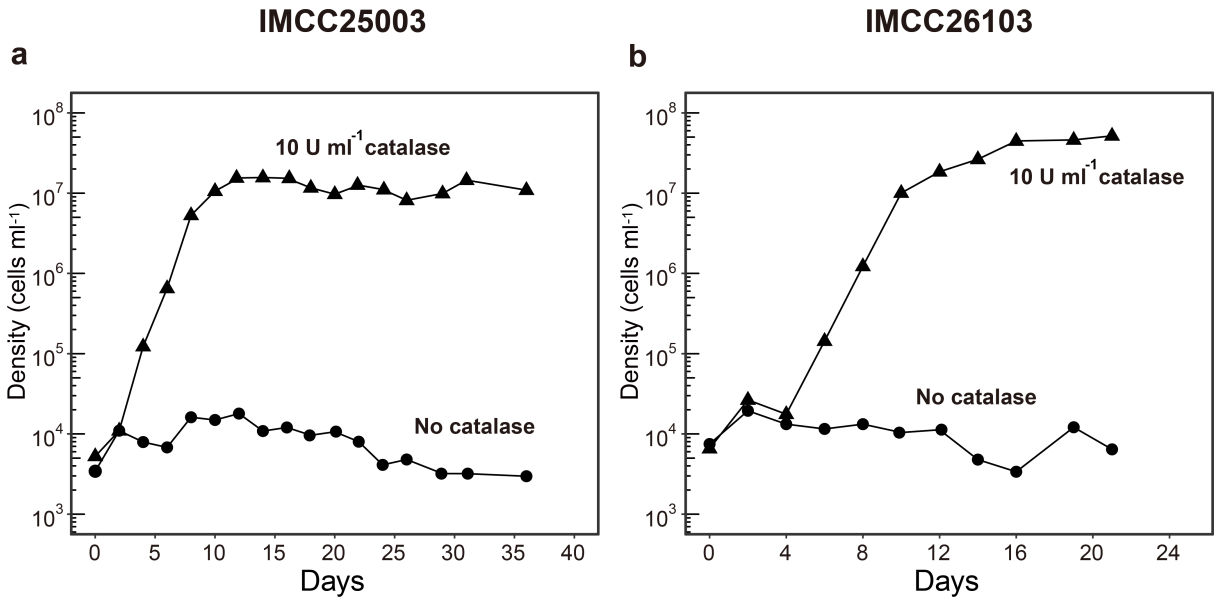
Supplementary Figures



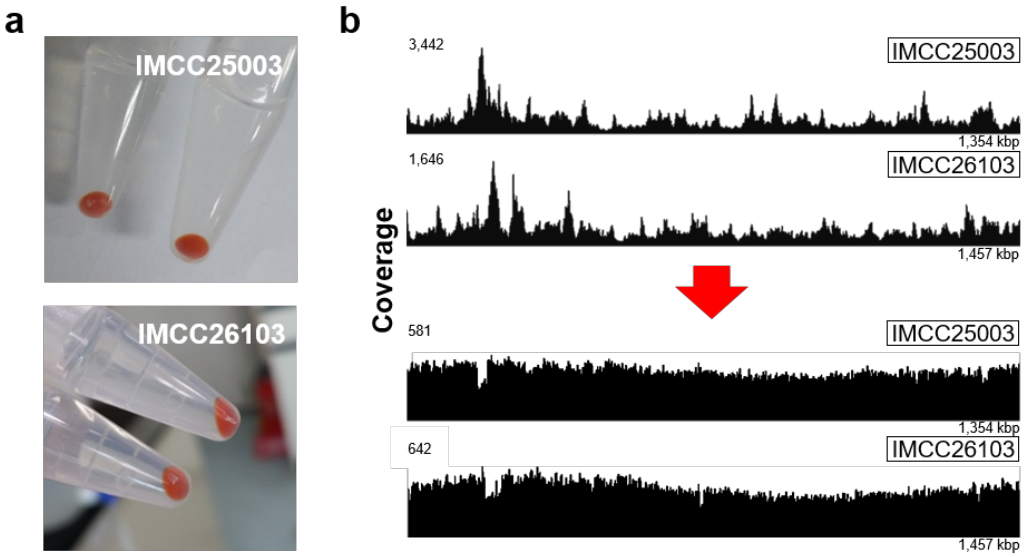
Supplementary Figure 1. Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic position of strains IMCC25003 and IMCC26103. The two strains isolated in this study are marked in red. *Streptomyces sannanensis* (AB184579) and *Streptomyces griseus* (AY999909) were used as outgroup. Bootstrap supporting values (from 600 replicates) are shown at the nodes as filled circles (≥90%), half-filled circles (≥70%), and empty circles (≥50%). Bar, 0.10 substitutions per nucleotide position.



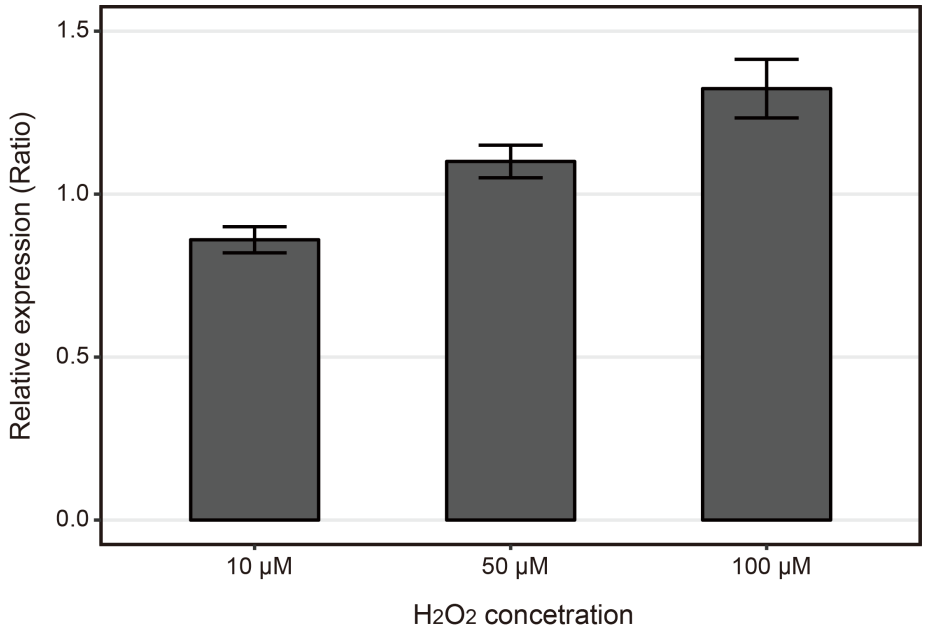
Supplementary Figure 2. Revival and transfer cultures of strains IMCC25003 and IMCC26103. (a) The growth curve of a revival culture of strain IMCC26103. (b) Flow cytometry plots of strain IMCC26103 obtained at the time points indicated in (a). Left, histograms showing the distribution of cell counts (y axis) according to the green fluorescence (x axis); Right, dot plots showing the distribution of cells according to side scatter (x axis) and green fluorescence (y axis). (c) Revival and two subsequent transfer cultures of strain IMCC25003.



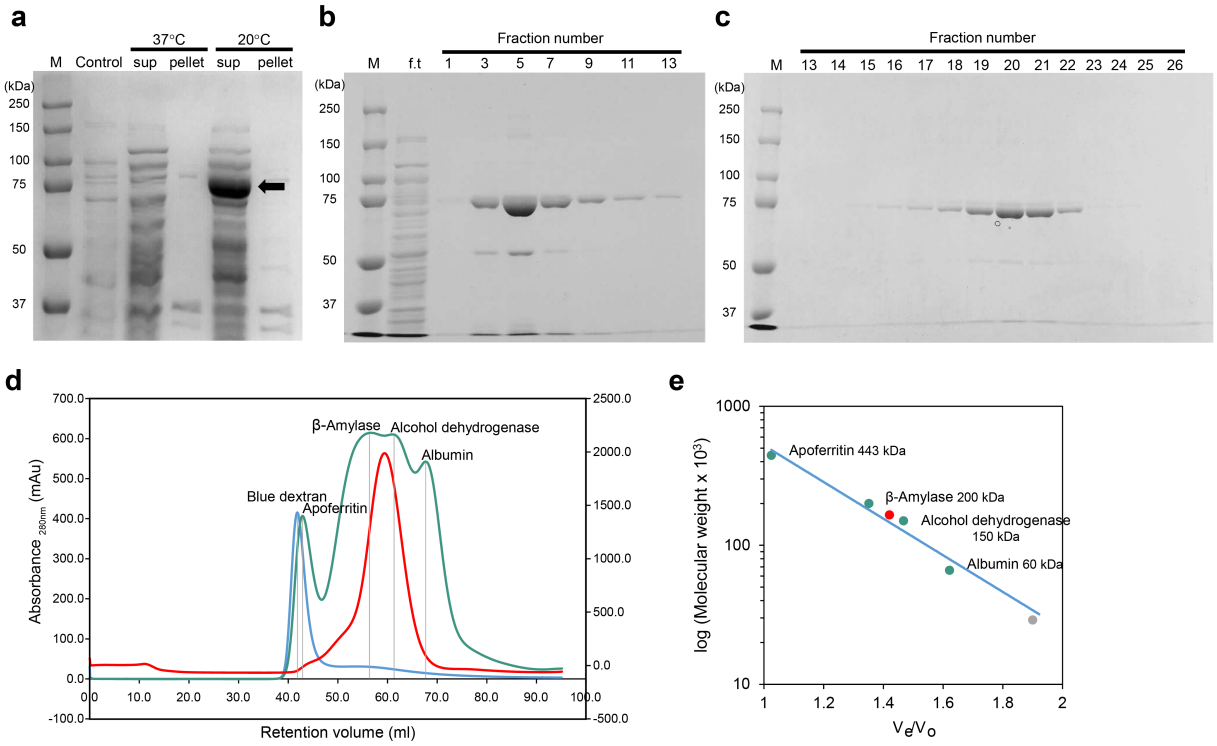
Supplementary Figure 3. Growth curves of strains IMCC25003 (a) and IMCC26103 (b) obtained from the revival experiment of frozen glycerol stocks using the culture medium (FAMV+CM+AA) supplemented with catalase. The triangle symbol represents growth in the medium amended with 10 U ml^{-1} catalase, and the circle symbol represents growth in the medium without catalase.



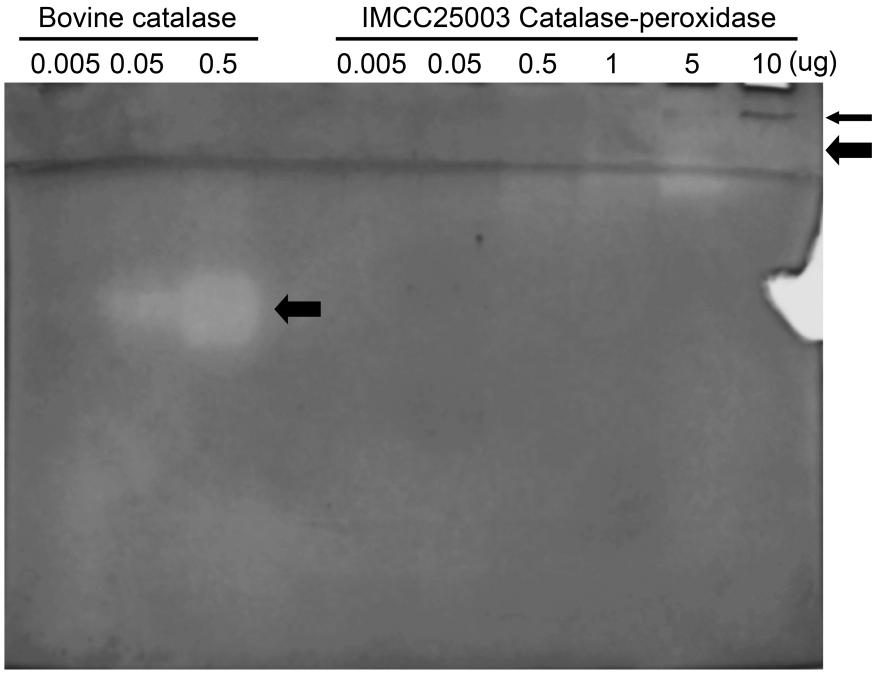
Supplementary Figure 4. Genome sequencing using the genomic DNA extracted from cultured and harvested cell pellets of the acI strains. (a) Cell pellets obtained by centrifugation from 4 L cultures of IMCC25003 (upper) and IMCC26103 (lower). Genomic DNA extracted from these cell pellets were used for genome sequencing. (b) Coverage variation across the complete genome sequences of the acI strains. The two coverage plots above red arrow were obtained from our previous study using whole genome amplification (WGA)[3]. The two coverage plots below the arrow were obtained from this study using large-scale cultures without WGA. Coverage variation was calculated using a 25-bp window based on read mapping. Bar heights were normalized in each plot and the maximum coverages are indicated at the upper left corner of each plot.



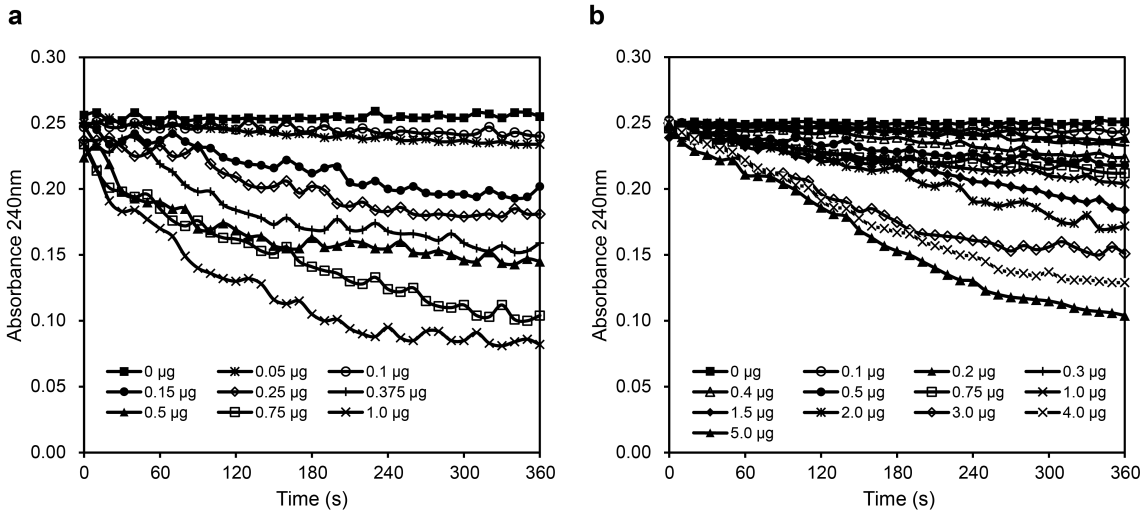
Supplementary Figure 5. Increase of IMCC25003 *katG* expression with increasing concentration of H_2O_2 treatment. Cells of IMCC25003 were treated with 3 different H_2O_2 concentrations (10 μM, 50 μM, and 100 μM) for 30 min and total RNA was used for the analysis of *katG* expression by qPCR. Expression level of *katG* in H_2O_2-treated cultures was compared with that in the control cultures (no H_2O_2 treatment). FAMV+CM+AA was used as the culture medium. Error bars indicate standard deviations (n=3).



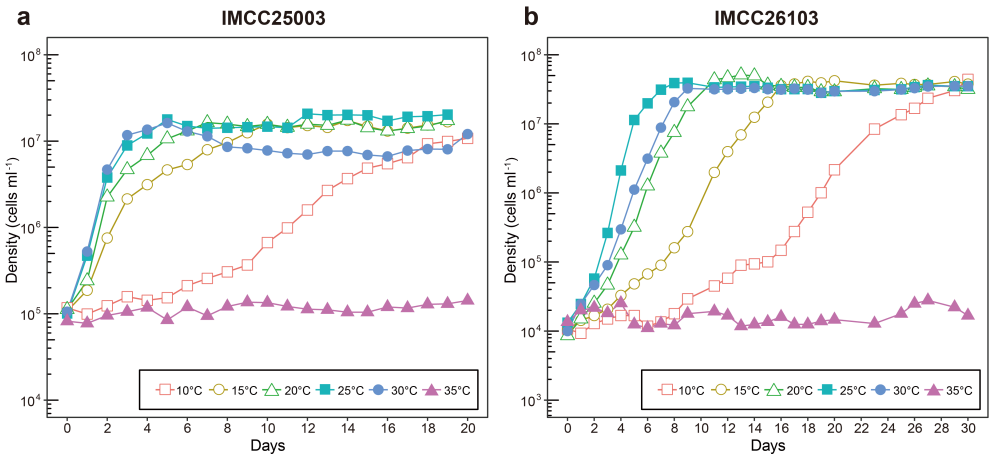
Supplementary Figure 6. Expression, purification, and determination of native molecular weight of recombinant IMCC25003 KatG. (a) Expression of IMCC25003 KatG in *E. coli* analyzed by SDS-PAGE. The bold arrow indicates a band of KatG, which is approximately 82.6 kDa. M, molecular weight size marker; control, before induction of expression; sup, supernatant; pellet, cell debris and membrane. Purified IMCC25003 KatG bound to a Ni^{2+}-nitrilotriacetic acid affinity column (b) and the purified protein through a size exclusion superpose-12 column (c), confirmed by SDS-PAGE. M, molecular weight size marker; f.t., unbound flow through fraction. Chromatograms (d) of protein-molecular-weight size markers and IMCC25003 KatG, and the molecular-weight calibration curve (e) obtained from protein-molecular-weight size markers and IMCC25003 KatG. The chromatogram colored in red and the red dot on the calibration curve represent IMCC25003 KatG.



Supplementary Figure 7. Catalase and peroxidase activities of purified IMCC25003 KatG and bovine catalase (KatE). Bovine catalase [0.005 (0.01 unit), 0.05 (0. 1 unit), and 0.5 (1 unit) μg] and IMCC25003 KatG (0.005, 0.05, 0.5, 1, 5, and 10 μg) of IMCC25003 KatG were separated by 8% non-denaturing PAGE. The bold arrows indicate negatively stained catalase activity and the narrow arrow indicates peroxidase activity stained by 3,3′,5,5′-tetramethylbenzidine.



Supplementary Figure 8. Kinetic curves of H_2O_2 decomposition by IMCC25003 KatG and bovine catalase. The curves of absorbance at 240 nm over time were generated using varying quantities of (a) bovine catalase (0–1.0 µg) and (b) IMCC25003 KatG (0 –5.0 µg).



Supplementary Figure 9. Growth curves of strain IMCC25003 (a) and strain IMCC26103 (b) at varying temperature conditions.

Supplementary Tables

Supplementary Table 1. Media used in this study and their composition.

Components of media		
Components (abbreviation)	Compound(s)	Final concentration
Ammonium (N)	NH_4Cl	10 μM
Phosphate (P)	KH_2PO_4	10 μM
Trace metals (TM)	$FeCl_3 \cdot 6H_2O$	117 nM
	$MnCl_2 \cdot 4H_2O$	9 nM
	$ZnSO_4 \cdot 7H_2O$	800 pM
	$CoCl_2 \cdot 6H_2O$	500 pM
	$Na_2MoO_4 \cdot 2H_2O$	300 pM
	Na_2SeO_3	1 nM
	$NiCl_2 \cdot 6H_2O$	1 nM
Vitamin mixture (V)	Thiamine·HCl	59 nM
	Niacin	81 nM
	Ca-Pantothenate	84 nM
	Pyridoxine	59 nM
	Biotin	409 pM
	Folic acid	453 pM
	Vitamin B12	70 pM
	Myo-inositol	555 nM
	p-Aminobenzoic Acid	7 nM
Carbon mixture (CM)	Pyruvate	50 μM
	D-Glucose	5 μM
	N-Acetyl-D-glucosamine	5 μM
	D-Ribose	5 μM
	Methyl alcohol	5 μM
20 proteinogenic amino acid mixture (AA)	Each amino acid	100 nM, each
Media definition		
Media	Definition	
FAM	0.2 μm-filtered and autoclaved freshwater medium supplemented with N, P, and TM	
FAMV	FAM supplemented with V	
FAMV+CM	FAMV supplemented with CM	
FAMV+AA	FAMV supplemented with AA	
FAMV+CM+AA	FAMV supplemented with CM and AA	

Supplementary Table 2. Trials to establish pure culture of strain IMCC25003.

Trial	Media composition	Additional substrate	Reference
1st attempt	FAMV		
	FAMV+CM		
	FAMV+AA	0.5×, 1×, 5×, and 10× of CM	
	AFM[a]+V+CM+AA		
	FM[b]+V+CM+AA		
2nd attempt	FAMV+CM+AA	20 μM acetate	[4]
		20 μM oxaloacetate	[5]
		20 μM putrescine	[5,6]
		20 μM glycerol	[5]
		20 μM xylose	[6,7]
		1 mg L^{-1} proteose peptone No. 3	
		1 mg L^{-1} yeast extract	
3rd attempt	FAMV+CM+AA	1:20 diluted spent medium[c]	[6,8], This study
4th attempt	FAMV+CM+AA	10 U ml^{-1} catalase	

[a]AFM, Artificial freshwater medium[9]. [b]FM, 0.1 μm-filtered but non-autoclaved freshwater medium. [c]Spent medium, a spent medium of the genus *Limnohabitans* filtrated through 0.1 μm pore-size membrane after cultivation of *Limnohabitans* sp. IMCC26003. For the media abbreviations, refer to Supplementary Table 1.

Supplementary Table 3. Kinetic parameters of various catalase-peroxidases and bovine catalase.

Source	Molecular weight	Structure	Specific activity (Units mg^{-1})	*K*m (mM)	*k*cat (s^{-1})	*k*cat/*K*m (M^{-1} s^{-1})	pI	Reference
IMCC25003[a]	165,000	A2	179.3	11.7	9.05×10^2	8.01×10^4	7.6[b]	This study
Archaeoglobus fulgidus[a]	NA	NA	5,280	3.8	7.77×10^3	2.04×10^6	5.6[b]	[10]
Bacillus selenatarsenatis SF-1	165,000	A2	3,375	2.6	1.15×10^4	4.41×10^6	6.0	[11]
Burkholderia pseudomallei[a]	NA	NA	3,630	4.5	5.68×10^3	1.26×10^6	5.9[b]	[10]
Escherichia coli K10	337,000	A4	1,486.5	3.9	1.63×10^4	4.19×10^6	5.1[b]	[12]
Escherichia coli O157:H7	NA	NA	NA	4.0	1.40×10^4	3.50×10^6	5.1[b]	[13]
Geobacillus stearothermophilus[a]	NA	NA	3,120	4.4	1.40×10^3	3.18×10^5	5.2	[14]
Halobacterium salinarum	240,000	A4	43.2	3.7	NA	NA	3.8	[15]
Mycobacterium smegmatis	NA	NA	NA	1.4	2.38×10^3	1.70×10^6	5.0[b]	[16]
Mycobacterium tuberculosis[a]	175,000	A2	2,420	5.2	1.01×10^4	1.94×10^6	5.1	[17]
Rhodobacter capsulatus	236,000	A4	7,800	4.2	NA	NA	4.5	[18]
Rhodobacter capsulatus[a]	NA	NA	4,830	3.7	6.64×10^3	1.79×10^6	5.1[b]	[10]
Synechococcus elongatus PCC 6301[a]	165,000	A2	1,491	4.8	8.85×10^3	1.84×10^6	4.6	[19]
Synechococcus elongatus PCC 6301	165,000	A2	NA	4.3	7.20×10^3	1.67×10^6	5.1[b]	[20]
Synechococcus elongates PCC 7942[a]	NA	NA	NA	4.2	2.60×10^4	6.19×10^6	5.1[b]	[21]
Synechocystis sp. PCC 6803[a]	170,000	A2	5,420	4.9	3.50×10^3	7.14×10^2	5.4	[22]
Thermoascus aurantiacus	330,000	A4	NA	48.0	1.07×10^5	2.22×10^6	4.5	[23]
Thermus brockianus	178,000	A4	5,300	35.5	6.00×10^3	1.69×10^5	4.7	[24]
Bos taurus[c]	240,000	A4	1980.3	20.6	9.05×10^4	8.01×10^4	5.4	This study

[a]Biochemical properties were determined using recombinant catalase-peroxidase. [b]Theoretical pI values were estimated based on amino acids sequences. [c]The monofunctional bovine catalase which was amended to culture media of IMCC25003 was used as an experimental positive control. NA, not available.

Supplementary Table 4. List of acI genomes used in this study and the presence or absence of *katG* gene.

Tribe	Organism name	Genome ID	Isolation site	Complete	No. of Scaffolds	Genome size (bp)	Length of KatG (aa)
A1	Actinobacteria bacterium IMCC25003	2602042019[a]	Lake Soyang	O	1	1,353,947	746
	actinobacterium SCGC AAA278-O22	2236661007[a]	Lake Mendota	X	43	1,138,490	X
	actinobacterium SCGC AAA027-M14	2236661003[a]	Lake Mendota	X	22	822,296	725
	'Ca. Planktophila dulcis' MMS-IIA-65	CP016777[b]	Lake Zurich	O	1	1,348,019	732
	'Ca. Planktophila dulcis' MMS-IA-53	CP016772[b]	Lake Zurich	O	1	1,365,934	732
	'Ca. Planktophila dulcis' MMS-21-155	CP016770[b]	Lake Zurich	O	1	1,361,776	732
	'Ca. Planktophila sulfonica' MMS-IA-56	CP016773[b]	Lake Zurich	O	1	1,344,614	747
	'Ca. Planktophila versatilis' MMS-IIB-76	CP016778[b]	Lake Zurich	O	1	1,325,420	733
	'Ca. Planktophila versatilis' MMS-IA-79	CP016774[b]	Lake Zurich	O	1	1,331,009	733
	'Ca. Planktophila versatilis' MMS-IA-105	CP016775[b]	Lake Zurich	O	1	1,326,591	733
	'Ca. Planktophila versatilis' MMS-IIB-142	CP016781[b]	Lake Zurich	O	1	1,266,983	733
A2	'Ca. Planktophila limnetica' MMS-VB-114	CP016782[b]	Lake Zurich	O	1	1,328,793	722
A4	Actinobacteria bacterium IMCC26103	2602042020[a]	Lake Soyang	O	1	1,456,516	X
	'Ca. Planktophila lacus' MMS-IIB-106	CP016780[b]	Lake Zurich	O	1	1,384,812	721
	'Ca. Planktophila lacus' MMS-IIB-60	CP016783[b]	Lake Zurich	O	1	1,410,107	721
	'Ca. Planktophila lacus' MMS-21-148	CP016769[b]	Lake Zurich	O	1	1,460,061	721
A5	actinobacterium SCGC AAA044-O16	2606217200[a]	NA	X	17	1,313,698	718
	actinobacterium SCGC AAA028-G02	2606217191[a]	NA	X	18	1,231,401	718
A6	actinobacterium SCGC AAA028-E20	2602042080[a]	NA	X	19	727,714	X
	actinobacterium SCGC AAA028-I14	2619618809[a]	NA	X	11	623,569	717
A7	Actinobacteria bacterium IMCC19121	2606217181[a]	Lake Soyang	O	1	1,506,415	X
	actinobacterium SCGC AAA044-N04	2236661005[a]	Damariscotta Lake	X	23	1,286,658	718
	actinobacterium SCGC AAA024-D14	2264265190[a]	Sparkling Lake	X	82	778,696	X
	actinobacterium SCGC AAA023-J06	2236661001[a]	Sparkling Lake	X	98	695,943	X
	'Ca. Planktophila vernalis' MMS-IIA-15	CP016776[b]	Lake Zurich	O	1	1,364,004	718

B1	actinobacterium SCGC AAA027-L06	2505679121[a]	Lake Mendota	X	75	1,163,583	X
	actinobacterium SCGC AAA027-J17	2236661002[a]	Lake Mendota	X	81	966,755	X
	actinobacterium SCGC AAA278-I18	2236661006[a]	Damariscotta Lake	X	54	944,397	X
	actinobacterium SCGC AAA028-A23	2236661004[a]	Lake Mendota	X	64	833,294	X
	actinobacterium SCGC AAA023-D18	2236661009[a]	Sparkling Lake	X	67	753,259	X
	actinobacterium SCGC AB141-P03	2236876028[a]	Lake Stechlin	X	66	660,403	X
	'Ca. Nanopelagicus limnes' MMS-21-122	CP016768[b]	Lake Zurich	O	1	1,238,108	X
	'Ca. Nanopelagicus hibericus' MMS-21-160	CP016771[b]	Lake Zurich	O	1	1,223,088	X
	'Ca. Nanopelagicus abundans' MMS-IIB-91	CP016779[b]	Lake Zurich	O	1	1,161,863	X
B4	actinobacterium SCGC AAA044-D11	2619618811[a]	NA	X	18	1,095,756	719
C1	Actinobacteria bacterium IMCC26077	2602042021[a]	Lake Soyang	O	1	1,551,612	X

[a]IMG Genome ID (IMG Taxon ID). [b]GenBank accession number. NA, not available.

Supplementary Table 5. Fatty acids composition (%) of two acI strains.

Fatty acid	IMCC25003	IMCC26103
Saturated fatty acids		
C10:0		0.46
C12:0	1.64	7.93
C14:0	18.22	7.85
C16:0	23.11	28.45
C17:0		1.05
C18:0	2.14	10.49
Unsaturated fatty acids		
C15:1 ω6*c*	1.11	
C17:1 ω8*c*	2.31	1.35
C18:1 ω9*c*	2.10	25.80
summed feature 3 (16:1 ω7*c*/16:1 ω6*c*)	45.79	12.28
summed feature 5 (18:2 ω6,9*c*/18:0 ante)		0.99
summed feature 8 (18:1 ω7*c*, 18:1 ω6*c*)	3.58	3.36

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