Supplementary Information

Unveiling the Repressive Mechanism of a PPS-like Regulator (PspR) in Polyhydroxyalkanoates Biosynthesis Network

Junyu Chen[1], Yinglu Cui[1,2], Shengjie Zhang[1], Bian Wu[1,2], Jing Han[1,2*], Hua Xiang[1,2*]

[1] State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, 100101, China

[2] College of Life Science, University of Chinese Academy of Sciences, 100049, Beijing, People's Republic of China

[*]Correspondence: hanjing@im.ac.cn (for J. Han) or xiangh@im.ac.cn (for H. Xiang)

Table S1 Strains and plasmids used in this study.

Strains	Relevant characteristics	Source or reference
Escherichia coli JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*	(Sambrook et al. 1989)
E. coli JM110	*dam dcm* mutant of *E. coli* JM109	Novagen
E. coli BL21 (DE3)	F^-ompT hsdS($r_B^-m_B^-$) *gal dcm*(DE3)	Novagen
H. mediterranei	*H. mediterranei* ATCC 33500	(Lu et al. 2008)
ΔEPS	EPS synthesis gene cluster deletion and *pyrF*-deleted mutant of wild-type strain *H. mediterranei* ATCC 33500	(Zhao et al. 2013)
ΔEPSΔ*pspR*	*pspR* deletion mutant of ΔEPS	(Chen et al. 2019)
ΔEPSΔ*pspR*(PspR-Myc)	overexpression of PspR with Myc-tag in ΔEPSΔ*pspR* strain	This study
ΔEPSΔRPEC::*gfp*	PHA related genes cluster ORF replaced by GFP ORF *in situ* in ΔEPS strain	This study
ΔEPSΔ*pspR*ΔRPEC::*gfp*	PHA related genes cluster ORF replaced by GFP ORF *in situ* in ΔEPSΔ*pspR* strain	This study
ΔEPSΔ*pspR*ΔRPEC::*gfp*(502)	ΔEPSΔ*pspR*ΔRPEC::*gfp* strain with pWL502 plasmid	This study
ΔEPSΔ*pspR*ΔRPEC::*gfp*(PspR)	overexpression of PspR in ΔEPSΔ*pspR*ΔRPEC::*gfp* strain	This study
ΔEPSΔ*pspR*ΔRPEC::*gfp*(E_{377}A-R_{519}A)	overexpression of mPspR (E_{377}A-R_{519}A) in ΔEPSΔ*pspR*ΔRPEC::*gfp* strain	This study
ΔEPSΔ*pspR*ΔRPEC::*gfp*(W_{360}A-W_{380}A)	overexpression of mPspR (E_{377}A-R_{519}A) in ΔEPSΔ*pspR*ΔRPEC::*gfp* strain	This study
Haloferax volcanii H1424	Δ*pyrE2* Δ*hdrB pitANph* Δ*mrr cdc48d-Ct*	(Stroud et al. 2012)
Plasmids	**Relevant characteristics**	**Source or reference**
pHFX	4.0 kb; integration vector containing *pyrF* and its native promoter, Amp^r	(Liu et al. 2011)
pHFX-GFP	5.2 kb; integration vector of pHFX for replacement of *gfp*	This study
pWL502	7.9 kb; shuttle vector with *pyrF* marker, Amp^r	(Liu et al. 2011)
pWL502-PspR	10.8 kb; overexpression of PspR	This study

pWL502-E_{377}A-R_{519}A	10.8 kb; overexpression of mPspR	This study
pWL502-W_{360}A-W_{380}A	10.8 kb; overexpression of mPspR	This study
pWL502-PspR-Myc	10.6 kb; overexpression of PspR by with Myc-tag	This study
pTA06	8.0 kb; expression vector with N-terminal His_6 tag and promoter P_{phaR}	(Liu et al. 2015)
pTA06-PspR	10.6 kb; expression plasmid of pTA06 containing *pspR*	This study
pTA06-F_{340}A	10.6 kb; expression plasmid of pTA06 containing mutant *pspR*	This study
pTA06-W_{360}A	10.6 kb; expression plasmid of pTA06 containing mutant *pspR*	This study
pTA06-W_{380}A	10.6 kb; expression plasmid of pTA06 containing mutant *pspR*	This study
pTA06-E_{377}A-R_{519}A	10.6 kb; expression plasmid of pTA06 containing mutant *pspR*	This study
pTA06-W_{360}A-W_{380}A	10.6 kb; expression plasmid of pTA06 containing mutant *pspR*	This study
pTA06-F_{340}A-W_{360}A-W_{380}A	10.6 kb; expression plasmid of pTA06 containing mutant *pspR*	This study

Table S2. Primers used in this study.

Primers	Sequence (5'-3')	Description
RPEC-NF	GCGTGGCGTGGATGAGATATCGAGCTCCTTCTGCGAACGTTTCGTC	Amplification of upstream and downstream flanking regions of *phaR-phaP-phaEC* genes with GFP inserted into pHFX to construct pHFX-GFP
RPEC-GFP-NR	GTTCTTCTCCTTTACTCATCTCCTAACTCGGTGTTGTACC	
RPEC-GFP-CF	GGATGAACTATACAAATAATCGTTTTTTCGACGTGAAAATCG	
RPEC-CR	TATAGGGAGAAGCTTGCATGCCCCGGCCTAATCGATAGC	
RPEC-GFP-F	CGAGTTAGGAGATGAGTAAAGGAGAAGAAC	
RPEC-GFP-R	CGTCGAAAAAACGATTATTTGTATAGTTCATCC	
ChIP-PspR-ORF-F	GACAACAACCCCCCATGGATCTGCTGATGCAATGTGGCTCACA	Amplification of *pspR* with Myc-tag inserted into

ChIP-PspR-ORF-Myc-R	GCAAGGGAACCGCACACAAGAAAACTCACAGATCCTCCTCGCTGATGAGCTTCTGCTCGTACCGTTCGGACCGGTCGAG [a]	pWL502 to construct pWL502-PspR-Myc
Myc-F	GAGCAGAAGCTCATCAGCGAGGAGGATCTGTGA	
Myc-R	TCACAGATCCTCCTCGCTGATGAGCTTCTGCTC	
ChIP-Myc-F	CGGTCCGAACGGTACGAGCAGAAGCTCATCAGC	
ChIP-Myc-R	CGCACACAAGAAAACGGTACCTCACAGATCCTCCTCGCTGAT	
phaR-Pro-F	GGTCGTTACTGCGGCTCT	qPCR analysis of P_{phaR} region fragment
phaR-Pro-R	CCACCTCGCATCGTTTGA	
phaC1-Pro-F	AGGTTCCACATCGTAATCTCG	qPCR analysis of P_{phaC1} region fragment
phaC1-Pro-R	GTAAACGGGTTCATGGTCAT	
gvpA -Pro-F	TGAATCGGGCTGAACCAT	qPCR analysis of P_{gvpA} region fragment
gvpA-Pro-R	TAGGCAGGGTTTGGTGGG	
kch-Pro-F	CGGAACCAGCCACTGAAC	qPCR analysis of P_{kch} region fragment
kch-Pro-R	CGCGTGGCTACCCATATT	
pspR-F	CGCCATATGAACGAGAGGGGAGACACGCCAG	Amplification of *pspR* inserted into pTA06 to construct pTA06-PspR
pspR-R	CGCGGATCCGTACCGTTCGGACCGGTCGAGG	
pspR-360WAF	CTCGTCGTAGACTGGGCGCGGGAACTGCTCAAT [b]	Amplification of mutant *pspR* inserted into pTA06 to construct pTA06-F_{340}A and pTA06-F_{360}A
pspR-360WAR	ATTGAGCAGTTCCCGCGCCCAGTCTACGACGAG	
pspR-WA380F	AACACCGAAGCACCGGCGCGGCGGTCGAAGCCGGC	Amplification of mutant *pspR* inserted into pTA06 to construct pTA06-W_{380}A
pspR-WA380R	GCCGGCTTCGACCGCCGCCGGTGCTTCGGTGTT	
pspR-EA377F	CGTTCAGGGAACACCGCGGGCACCGTGGGCGGTC	Amplification of mutant *pspR* inserted into pTA06 to construct pTA06-E_{377}A
pspR-EA377R	GACCGCCCACGGTGCCGCGGT	

	GTTCCCTGAACG	
pspR-RA519F	TTGACAGTACTCAGTGCGTTACTCCCCCGTATC	Amplification of mutant *pspR* inserted into pTA06 to construct pTA06-$R_{519}A$
pspR-RA519R	GATACGGGGGAGTAACGCACTGAGTACTGTCAA	

[a] Underlines stands for Myc sequence.

[b] Double underlines stand for protein mutant sites.

Table S3 DNA enrichment assay of the PspR-Myc fusion protein.

Ct	PphaR			PphaC1			PgvpA			Pkch			7S		
output	26.51	26.93	26.97	26.83	26.92	26.99	26.35	26.78	27.29	25.44	25.45	26.92	25.82	25.97	26.31
mock	27.83	27.83	28.69	26.73	27.07	28.23	26.45	26.49	26.5	25.58	25.82	26.06	25.93	25.94	26.22
input	11.1	11.16	11.32	11.25	11.3	11.38	11.24	11.41	11.49	11.17	11.28	12.33	10.99	11.02	11.03

	PphaR			PphaC1			PgvpA			Pkch			7S		
$\Delta Ct_{output}=Ct_{output}-Ct_{input}$	15.41	15.77	15.65	15.58	15.62	15.61	15.11	15.37	15.8	14.27	14.17	14.59	14.83	14.95	15.28
$\Delta Ct_{mock}=Ct_{mock}-Ct_{input}$	16.73	16.67	17.37	15.48	15.77	16.85	15.21	15.08	15.01	14.41	14.54	13.73	14.94	14.92	15.19
$\Delta\Delta Ct=\Delta Ct_{output}-\Delta Ct_{mock}$	-1.32	-0.9	-1.72	0.1	-0.15	-1.24	-0.1	0.29	0.79	-0.14	-0.37	0.86	-0.11	0.03	0.09
$-\Delta\Delta Ct$	1.32	0.9	1.72	-0.1	0.15	1.24	0.1	-0.29	-0.79	0.14	0.37	-0.86	0.11	-0.03	-0.09
$2^{(-\Delta\Delta Ct)}$	2.496661	1.866066	3.294364	0.933033	1.109569	2.361985	1.071773	0.817902	0.578344	1.101905	1.292353	0.550953	1.079228	0.97942	0.939523
mean, SD	2.552364	0.715776		1.468196	0.779061		0.822673	0.246749		0.981737	0.385031		0.99939	0.071962	

Table S4 GFP expression is upregulated in ΔEPSΔ*pspR* ΔRPEC::*gfp* strain

	Exponential phase						Stationary phase					
Strain	**ΔEPSΔRPEC::*gfp***			**ΔEPSΔ*pspR*ΔRPEC::g*fp***			**ΔEPSΔRPEC::*gfp***			**ΔEPSΔ*pspR*ΔRPEC::g*fp***		
OD600	0.276	0.266	0.271	0.288	0.283	0.294	1.882	1.856	1.853	2.007	2.009	2.018
488,509	1142	1152	1145	3814	3891	3920	18839	19036	18028	22560	22157	22311
RFU	4137.681	4330.827	4225.092	13243.0556	13749.117	13333.333	10010.1	10256.47	9729.088	11240.6577	11028.87008	11055.996
Mean, SD	4231.2	96.71771		13441.8352	269.91468		9998.55	263.8783		11108.50794	115.2459254	
Fold change				3.17683746						1.111011923		

Table S5 Calculation of DNA-binding efficiency of mPspR to P_{phaR}.

Mutation site	Fig. 5a	Fig. 5b	Fig. 5c	Average[a]
PspR (wt[b])	1	1	1	1
$E_{377}A$-$R_{519}A$[c]	0.63	0.47	0.45	0.52 ± 0.09***
$W_{340}A$	0.75	0.45	0.69	0.63 ± 0.16**
$W_{360}A$	1.85	1.02	1.55	1.47 ± 0.17
$W_{380}A$	1.96	1.11	1.4	1.49 ± 0.19
$W_{360}A$-$W_{380}A$	1.75	1.77	1.65	1.72 ± 0.06**
$W_{340}A$-$W_{360}A$-$W_{380}A$	1.63	1.76	1.47	1.62 ± 0.15***

[a]All data are expressed as means ± standard deviations of independent repeated experiments results. Statistical significance is defined as ***$P < 0.001$, and **$P < 0.01$.

[b]wt is the wild type of PspR.

[c]Number is the mutation site of PspR.

Table S6 Different binding specificity of mPspRs to P_{phaR} region determined by fluorescent reporter system.

Strain	ΔEPSΔpspRΔRPEC::gfp(502)			ΔEPSΔpspRΔRPEC::gfp(PspR)			ΔEPSΔpspRΔRPEC::gfp(E377A-R519A)			ΔEPSΔpspRΔRPEC::gfp(E377A-R519A)		
OD600	0.257	0.264	0.272	0.276	0.268	0.272	0.264	0.277	0.271	0.269	0.262	0.261
488,509	3808	3938	3793	1155	1212	1052	2188	2112	2128	905	787	898
RFU	14817.1206	14916.6667	13944.85294	4184.782609	4522.38806	3867.647059	8287.878788	7624.548736	7852.398524	3364.312268	3003.816794	3440.613027
Mean, SD	14559.5467	534.662236		4191.605909	327.4238272		7921.608683	337.037422		3269.580696	233.2987147	
Fold change				**contrast=1**			1.889874395			0.780030558		

Figure

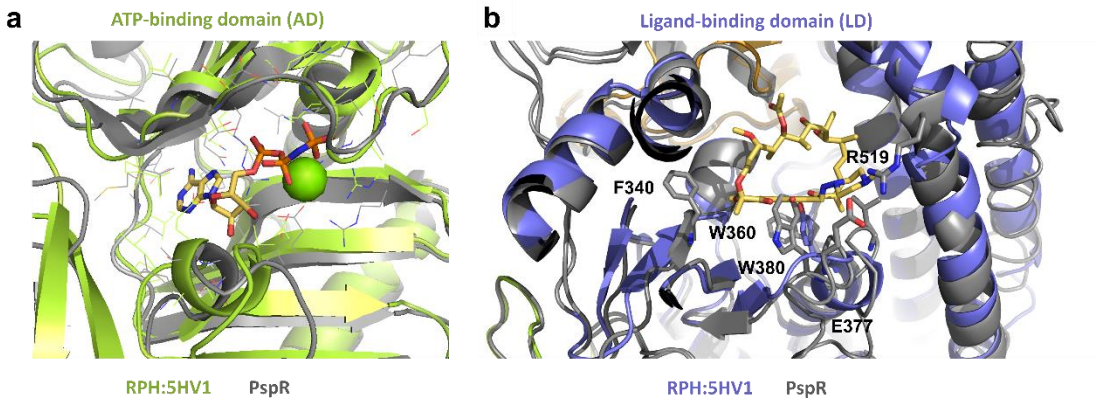

Fig. S1 Structure superimposition of predicted PspR model with RPH$_{Lm}$-rifampin bound complex (PDB ID: 5HV1). (a) Structure superimposition of ATP-binding domain (AD) in PspR model with RPH-ATP bound complex. PspR model and RPH-rifampin complex are colored grey and green, respectively. ATP is shown as stick; Mg^{2+} is shown as a green sphere. (b) Structure superimposition of Ligand-binding domain (LD) in PspR model with RPH-rifampin bound complex. PspR model and RPH-rifampin complex are colored grey and hyacinth, respectively. Rifampin is shown as stick. Candidate key residues in LD region are shown, W: tryptophan, R: arginine, E: glutamic acid.

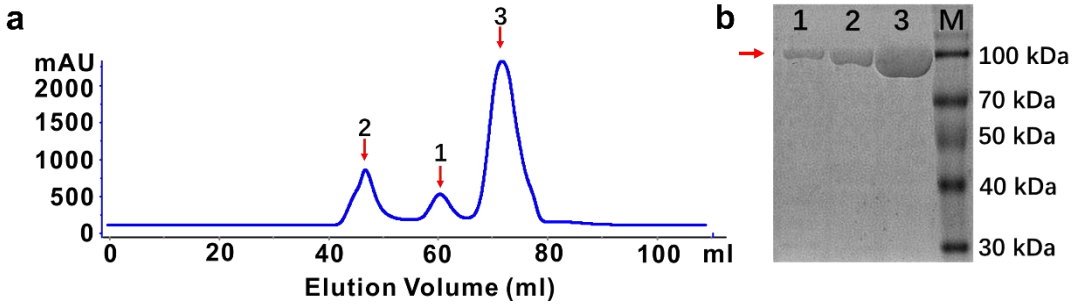

Fig. S2 Purification of RPH$_{Lm}$ expressed in by MS (a) and SDS-PAGE result (b). Both in (A) and (B), lane 3 is RPH protein monomer; lane 1 and 2 are different polymerization states.

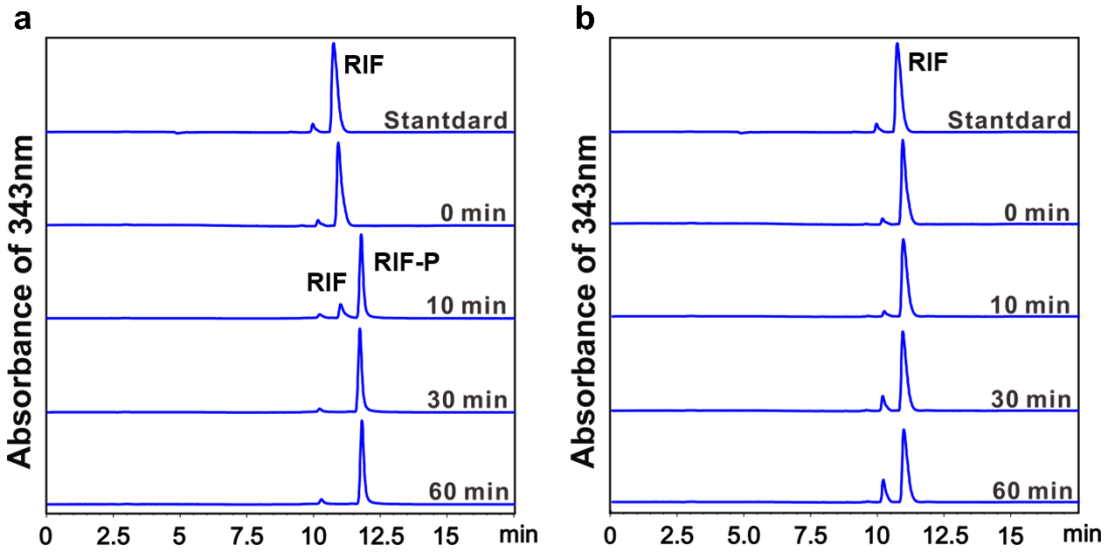

Fig. S3 Detection of phosphotransferase activity of RPH*Lm* (a) and PspR (b) by HPLC. Rifampin (RIF) is the substrate, and phosphorylated rifampin (RIF-P) is the product. HPLC was used to detect the substrate (RIF) and product (RIF-P) during the reaction at 0 min, 10 min, 30 min and 60 min time points. The retention time of RIF is about 11 min, and the retention time of RIF-P is about 12 min.

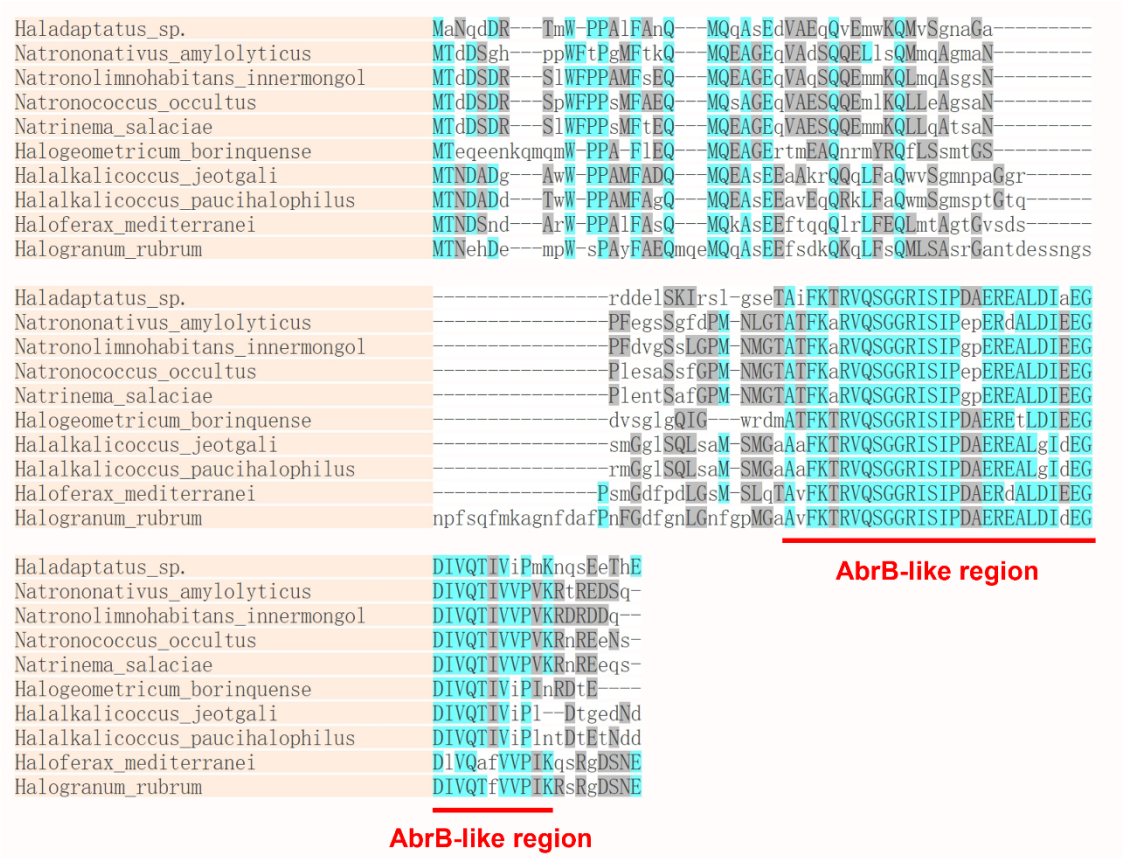

Fig. S4 Multiple alignments of amino acid sequences of PhaR homologs from nine representative haloarchaea species: *Haloferax mediterranei*, *Natrononativus amylolyticus*, *Natronolimnobius innermongolicus*, *Natronococcus occultus*, *Haladaptatus* sp., *Halogranum rubrum*, *Halogeometricum borinquense*, *Halalkalicoccus jeotgali*, *Halalkalicoccus paucihalophilus* and *Natrinema salaciae*. The AbrB-like region is indicated by red bar. Sequence analysis and database. Query sequences were accessed from the National Center for Biotechnology Information (NCBI) Protein Database. Sequence homology was analyzed *via* the Uniprot BLAST server (https://www.uniprot.org/). The sequences of each PhaR homolog are listed as follows:

>WP_004056141.1 *Haloferax mediterranei*

MTNDSNDARWPPALFASQMQKASEEFTQQQLRLFEQLMTAGTGVSDSPSMG DFPDLGSMSLQTAVFKTRVQSGGRISIPDAERDALDIEEGDLVQAFVVPIKQSR GDSNE

>WP_255167741.1 *Natrononativus amylolyticus*

MTDDSGHPPWFTPGMFTKQMQEAGEQVADSQQELLSQMMQAGMANPFEGS
SGFDPMNLGTATFKARVQSGGRISIPEPERDALDIEEGDIVQTIVVPVKRTREDS
Q

>WP_007261346.1 *Natronolimnohabitans innermongolicus*

MTDDSDRSLWFPPAMFSEQMQEAGEQVAQSQQEMMKQLMQASGSNPFDVG
SSLGPMNMGTATFKARVQSGGRISIPGPEREALDIEEGDIVQTIVVPVKRDRDD
Q

>WP_015320638.1 *Natronococcus occultus*

MTDDSDRSPWFPPSMFAEQMQSAGEQVAESQQEMLKQLLEAGSANPLESASS
FGPMNMGTATFKARVQSGGRISIPEPEREALDIEEGDIVQTIVVPVKRNREENS

>WP_066146951.1 *Haladaptatus* sp.

MANQDDRTMWPPALFANQMQQASEDVAEQQVEMWKQMVSGNAGARDDEL
SKIRSLGSETAIFKTRVQSGGRISIPDAEREALDIAEGDIVQTIVIPMKNQSEETH
E

>WP_241211152.1 *Halorubrum lacusprofundi*

MNTKNGLRNSKKRLKQRKTNSTNIAVILITYYFISPSRDMPRITTKGQVTIPKEI
RETLGIEPGDEIAFEEVSSGYKIQKKEPTTADGNDPFAKYRGSAESDETMPER
MRRLRREYPRDVGDDCDDESEAEA

>WP_089865813.1 *Halogranum rubrum*

MTNEHDEMPWSPAYFAEQMQEMQQASEEFSDKQKQLFSQMLSASRGANTDE
SSNGSNPFSQFMKAGNFDAFPNFGDFGNLGNFGPMGAAVFKTRVQSGGRISIP
DAEREALDIDEGDIVQTFVVPIKRSRGDSNE

>WP_006056100.1 *Halogeometricum borinquense*

MTEQEENKQMQMWPPAFLEQMQEAGERTMEAQNRMYRQFLSSMTGSDVSG
LGQIGWRDMATFKTRVQSGGRISIPDAERETLDIEEGDIVQTIVIPINRDTE

>WP_008417219.1 *Halalkalicoccus jeotgali*

MTNDADGAWWPPAMFADQMQEASEEAAKRQQQLFAQWVSGMNPAGGRSM
GGLSQLSAMSMGAAAFKTRVQSGGRISIPDAEREALGIDEGDIVQTIVIPLDTG
EDND

>WP_066382202.1 *Halalkalicoccus paucihalophilus*

MTNDADDTWWPPAMFAGQMQEASEEAVEQQRKLFAQWMSGMSPTGTQRM
GGLSQLSAMSMGAAAFKTRVQSGGRISIPDAEREALGIDEGDIVQTIVIPLNTD
TETNDD

>WP_090612128.1 *Natrinema salaciae*

MTDDSDRSLWFPPSMFTEQMQEAGEQVAESQQEMMKQLLQATSANPLENTS
AFGPMNMGTATFKARVQSGGRISIPGPEREALDIEEGDIVQTIVVPVKRNREEQ
S

Supplementary Reference

Chen JY, Mitra R, Zhang SJ, Zuo ZQ, Lin L, Zhao DH, Xiang H, Han J (2019) Unusual phosphoenolpyruvate (PEP) synthetase-like protein crucial to enhancement of polyhydroxyalkanoate accumulation in *Haloferax mediterranei* revealed by dissection of PEP-pyruvate interconversion mechanism. Appl Environ Microbiol 85(19) https://doi.org/10.1128/AEM.00984-19

Liu GM, Hou J, Cai SF, Zhao DH, Cai L, Han J, Zhou J, Xiang H (2015) A patatin-like protein associated with the polyhydroxyalkanoate (PHA) granules of *Haloferax mediterranei* acts as an efficient depolymerase in the degradation of native PHA. Appl Environ Microbiol 81(9):3029-38 https://doi.org/10.1128/aem.04269-14

Liu HL, Han J, Liu XQ, Zhou J, Xiang H (2011) Development of *pyrF*-based gene knockout systems for genome-wide manipulation of the archaea *Haloferax mediterranei* and *Haloarcula hispanica*. J Genet Genomics 38(6):261-9 https://doi.org/10.1016/j.jgg.2011.05.003

Lu QH, Han J, Zhou LG, Zhou J, Xiang H (2008) Genetic and biochemical characterization of the poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) synthase in *Haloferax mediterranei*. J Bacteriol 190(12):4173-80 https://doi.org/10.1128/JB.00134-08

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y

Stroud A, Liddell S, Allers T (2012) Genetic and biochemical identification of a novel single-stranded DNA-binding complex in *Haloferax volcanii*. Front Microbiol

3(224) https://doi.org/10.3389/fmicb.2012.00224

Zhao DH, Cai L, Wu JH, Li M, Liu HL, Han J, Zhou J, Xiang H (2013) Improving polyhydroxyalkanoate production by knocking out the genes involved in exopolysaccharide biosynthesis in *Haloferax mediterranei*. Appl Microbiol Biotechnol 97(7):3027-36 https://doi.org/10.1007/s00253-012-4415-3

3