## Supplementary Material

			10	20		30		40		50	ť	60	70		80		90		100	110	
E_coll/1-426 Bacillus subtilis/1-334	1					MFDR	YDAGEC	AVLVH	IYFTQD	KD	-MEDL	Q <mark>ef</mark> esi Eelasi	VSSAG-				VEALQ GKVLT	V I T <mark>g</mark> sr Sv tokr	KAPHPKY	FVGEGKAVE	66 40
Chlamydophila_pneumoniae/1-472	1	MDT	IDT <mark>PG</mark> E	QGSQSF	NSLGA	RFDL PR	KEQDPS	QAL AV	ASYQNK	TDSQ	VEEHLI	D <mark>el Is</mark> l	AD <mark>SC</mark> G-				ISVLE	TRSWIL	KTPSAST	Y INV <mark>GK</mark> LEE	1 93
Oryza_sativa/1-568 Homo_saniens/1_403	1	MLRAAVSRI		I <mark>PHP</mark> HAHI	HHGLE	SVTPAP	PRDPER	PPRLLY	VVQ <mark>P</mark> RL	RPGSL		AEAL NL		PRDGF	YKE <mark>g</mark> l A	AKGAP		QNPSSF TMVVST			/ 115 
Sulfolobus_solfataricus_/1-364	1								MKT	AAL F\	SKEFE	EEAIAL	VEGAN-				-YKVT	SIYKL	KSPNVKF	Y IQYDKLQQ	1 51
		120		130	140		150		160		170	1	80	190		200		<b>210</b>	2	20	
E_coll/1-426	67	AEAVKATG/	AS	VVL FDHA	AL S <mark>P</mark> AQ	E <mark>RNLE</mark> R	LCE-CR	V I DR T	SL I L D I	F AQR /	RTHEG	KL <mark>Q</mark> VEL	. A <mark>Q</mark> L R HL	AT <mark>R</mark> LV	RGWT <mark>H</mark> -		• • • • •	LE <mark>F</mark>	( KGG I G -	LRGPCE	<mark>r</mark> 158
Becillus_subtilis/1-334	41	KALVEELE/	AD		EL SPSQ		AIE-VK	MIDRT			RTREG	KLQIEL						LSF	GGGGIG-	ARGPCE	132
Chiamydophita_pheumoniae/1-472	94 116	KCYLETSE!	SEEGVE		TGIO		AWG-KP	VLDRV		F S SRA	ETKEA	KLOSEL	AUARTL				GSSGE	AEVVSA	GRGSGC	RGELSGAGE	225
Homo_sepiens/1-403	49	TEKIRGSPI	DVT0			KELEA	AWG - VE	VFDRF	TVVLHI	FRCNA	RTKEA	RLQVAL		RSNLK	RDVAH-			LY	TGVGSR-	YIMGSCE	5 142
Sulfolobus_solfataricus_/1-364	52	KNDEE	\$	S <mark>TLII</mark> FEQ	2 <mark>l kp</mark> rh	F I <mark>n i</mark> rr	RELKGKE	VL <mark>DK</mark> II	LLLEI	FALH/	GSKEA	KMQ I EL	ARLKYE		ET <mark>y</mark> tk-			<mark>S</mark> k	GEQQG-	PLGACT	Y 140
			240	25	0	260		270	-	280	G1	290	300	)	G2 310		320		Glycine 330 G3	rich region 340	
E_coli/1-426	159	QLETI RRLI		QIQSRLE	ER VE <mark>K</mark> Q	REQGRO	S <mark>r   K/</mark> D		• V <mark>PT</mark> VS		NAGKS	T_FNR	I <mark>T</mark> -EARV	YAADQ	FATLD	PTLRR		V <mark>GE</mark> TVL	DTVG I	RHL <mark>PH</mark> DLVA	A 266
Bacillus_subtilis/1-334	133	KLETI RRH		IE I NTQVS	AVIRH	RSRYRE					NAGKS	TVFNRL	T-SADS	YEEDL	FATLD	PMTRK	MVL PS	GYSVLL	SDTVGFI	QDLPTTLIA	A 240
Orvza sativa/1-568	230	ELQLORR	IQECR	RLLAQIE	DVRRT	RAIORS		NSFGQ	ELVTVA		NAGKS	TLVSAL	S-ETDL	YSDDR	FATVD	PRLES	VILPS	GRKALL	SDTVGFI	SDL PVQLVE	A 343
Homo_sapiens/1-403	143		LREKE/	KIRKAL	RLRKK	RHLLRR	QRTRF E		FPVIS	V' <mark>GY</mark> T	NC <mark>GK</mark> T	T . I KAL	TGDAAI	QPRDQ	FATLD	TAHA	GTL PSI	RMTVLY	' <mark>NDT IG</mark> FL	SQL PHOL I E	<mark>5</mark> 251
Sulfolobus_solfataricus_/1-364	141	GVES I KEY	YKRRII		SIKIF	KEKSIE	SNK <mark>r</mark> i N		I PSIG	I GYT	NSGKT	S_FNSL	. T - GL TC	KVDTK	FTTMS	PKRYA	I P I NN	-RK <mark>IM</mark> L	DTVG	R <mark>G   PP</mark> Q   VD.	A 247
		350	Arg	360	egion 370		380		390		400 G4	4	10	420	8	430		440	4	50	-
E_coll/1-428	267	FKATLQET	RQATLI	L HV I DAA	DVR	VQ <mark>E</mark> N I E	AVNT VL	<mark>ee</mark> Idal	4E I	PTLL\	TIN <mark>K</mark> I D	ML ED F	E PR I DR	DE					ENK	PNRWL SAQ	Г 346
Bacillus_subtilis/1-334	241	FRSTLEEVI	KEADL	IL HL IDSS	NED			EELEAD	DD		NKRD	KL PDF							<mark>F</mark>	PEGITLWSV	₹ 313
Orvza sativa/1-568	344	FHATLEEV			APN	IEEHRS	TVLOVL			SMIE		VDNNE		EDEI	FLTEGE	EDKEE	ELESE		ESSFESL	DDGTDSEYL	S 456
Homo_sapiens/1-403	252	FSATLEDV/	AHS <mark>DL</mark>		SH <mark>P</mark> E,	AELQKO	SVLSTL	RGLQL	PAPLLD	SMVE		VPGYS	8 <mark>P</mark> T						E	PNVVPVSAL	R 327
Sulfolobus_solfatarious_/1-364	248	FFVTL SEA	KYS <mark>DAL</mark>	ILVI <mark>d</mark> s <mark>1</mark>	FSENL		SSFEIL	REIGV	S <mark>G</mark> K	PILV	NKID	( I N <mark>g</mark> dl		VEK					•••••L	SKELYSPIF	) 329
			470	48	0	490		500		510		520	530	)	540		550		560	570	Č.
E_co#/1-426	347	GAGI PQL FO	QAL TEF	RES <mark>G</mark> EVAC		PPQEGR	LRSR	FYQLQ/	AIEKEW	MEED	SVSLQ	VRM <mark>P</mark> I \	/DWRRLC	KQE <mark>P</mark> A	L IDYL I						426
Bacillus_subtilis/1-334	314		Q <mark>PL</mark> KKF	RESAICAN	(NC											DCD00					334
Chiamyoophila_pheumonilae/1-472 Orvza_sativa/1-568	3/8		GELSSS	SLE <mark>P</mark> SEMF		SSSSKD	CF <mark>G</mark> E		TISTDS	CSSTC		TL <mark>P</mark> ···			L IDKKL	TERQT	IVERS	GPVLES Y <mark>gp</mark> fdr	KWRPSSS		4/2
Homo_sapiens/1-403	328	GHGLQELK/	AELDA	VLKATG			AQL SWL	YKEAT	VQEVDV	IPED	AADVR	VIISNS	SAYGKER	KLFPG							403
Sulfolobus_solfataricus_/1-364	330	VIPISALK	RTNLE	LRDK I YO		SLE															358

**Figure S1**: *In-silico* analysis of HflX homologues reveals significant sequence features. Protein sequences belonging to the HflX family, present in all bacteria, eukaryotes, archea but absent in some classes like Mycoplasma, the epsilon subdivision of Proteobacteria, spirochaetes, the archaeon Methanobacterium and fungi, were retrieved from NCBI database and aligned using ClustalX [1]. A Multiple sequence alignment (MSA) of representative sequences, modified manually using Jalview alignment editor [2], is shown in the figure. Domain analysis showed a well defined G domain (region underlined) containing the conserved sequence motifs G1-G4 (boxed), known to be required for GTP binding. Preceding the G domain is a conserved N terminal domain of ~190 amino acids that has no significant sequence similarity to any well characterized domain. It contains a glycine rich region (boxed), which appears to be unique to Obg and HflX families. Sandwiched between the glycine rich region and the G domain, is an arginine rich region (boxed), which presents a positively charged surface that may perhaps serve as a binding site for the rRNA that HflX interacts with. C-terminal domain that follows the G domain (363-426) is not present in some of the orthologs. The numbers refer to the residue numbers in *E. coli* HflX.



**Figure S2**: Co-fractionation experiments, as in Fig 3C, were repeated with purified HfIX and the 30S subunit in presence of various nucleotides (shown in separate panels), as indicated on the right. Like in Fig 3C, the top fractions devoid of 30S and only the peak fractions containing 30S are shown (top gel, in blue color) based on the presence of 16S rRNA in these fractions (lower gel in grayscale). HfIX does not co-fractionate with the 30S, in any of the conditions tested.

## **Cloning, Expression and Purification of HflX**

*E. coli* hflX gene was amplified and cloned in pET28a vector (Novagen) using primers, given in table S1 (see below), containing BamHI and XhoI restriction sites. For overexpression of recombinant proteins, *E. coli* BL21 cells containing the recombinant plasmid was grown at 37°C and was induced with 0.4mM IPTG (Sigma-Aldrich) at 0.6 OD<sub>600</sub>. Culture was harvested by centrifugation at 4000 X g at 4°C for 10 minutes, after 8 hours of incubation at 25°C.

Similarly  $\Delta$ N-HflX lacking 192 amino acids at the N-terminal,  $\Delta$ C HflX lacking 62 amino acids at the C-terminal and HflX-G (amino acids 193-362) constructs were cloned in pQE2 vector (Qiagen) with NdeI and HindIII restriction sites using appropriate primers (see table S1). DH5 $\alpha$  cells carrying the recombinant plasmid were grown at 37°C and induced at 0.6 OD<sub>600</sub> with 0.2mM IPTG for over-expression. Cultures were harvested by centrifugation at 4000 X g at 4°C for 10 minutes after 12 hours of incubation at 17°C.

Cell pellet was lysed by 5 cycles of freeze-thaw in lysis buffer A {20mM Tris-HCl pH 8, 500mM NaCl, 5% Glycerol, 1mg/ml lysozyme, 3mM  $\beta$ -mercaptoethanol, 1mM PMSF, protease inhibitor cocktail (Sigma-Aldrich)}. This was followed by DNase and RNase treatment. Lysates thus obtained were centrifuged at 35,000 X g at 4°C for 1hour in 50mL oak-ridge tubes (Sorvall SS-34 rotor). Clarified supernatant was loaded on a 5mL Global His-trap affinity column (Amersham) equilibrated with buffer B (20mM Tris-HCl pH 8.0 at 4°C, 500mM NaCl, 5% glycerol 3mM  $\beta$ -meracptoethanol). The column was washed with 100mL of washing buffer B. A linear gradient of imidazole (0-500mM, in buffer B) was used to elute the protein. Eluted fractions were analyzed by SDS PAGE and concentrated with Millipore Amicon ultra centrifugal filter tubes (30KDa cutoff).

Concentrated protein sample was further purified by size exclusion chromatography using superdex200 column (Amersham). The protein eluted as a monomer. Fractions containing the protein were concentrated using Millipore Amicon ultra centrifugal filter tubes (10KDa cutoff), aliquoted and stored at -80°C after snap freezing in liquid nitrogen.

S.No	Oligo Name	Restriction	Sequence (5'-3')						
		site <sup>§</sup>							
1	HflX1S*	BamHI	GCGGAT <u>GGATCC</u> TTGTTTGACCGTTATGA						
2	HflX1S*	NdeI	CGC <u>CATATG</u> TTGTTTGACCGTTATGATGC						
3	HflX193S	NdeI	CGC <u>CATATG</u> ATCAAGCCGACGTTCCTAC						
4	HflX 362A*	HindIII	CCC <u>AAGCTT</u> CTAAAGCCGCTTCCGTCAAAGC						
5	HflX426A*	HindIII	CGC <u>AAGCTT</u> CTAGATCAGGTAATCGATCAA						
6	HflX426A*	XhoI	GCTCTGC <u>CTCGAG</u> CGTTAGATGAGGTAATC'						

**Table S1:** Oligos used for cloning the HflX constructs

\*Sense and antisense strands are indicated by S and A, respectively.

<sup>§</sup> Restriction sites used for cloning (underlined in sequence).

Numbers (in the oligo names) indicate the amino acid in the primary sequence of E. coli HflX.

## **References:**

- [1]. J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins, The CLUSTAL\_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 25 (1997) 4876-4882.
- [2] M. Clamp, J. Cuff, S.M. Searle, and G.J. Barton, The Jalview java alignment editor. Bioinformatics 20 (2004) 426-427.