Supplemental Information:

Supplemental Materials and Methods:

In vitro cross-linking of CtsR

CtsR was incubated at different temperatures with or without an excess of *clpC* promoter fragment and cross-linked by the addition of 2mM EDC and 5mM NHS for 20 min. (Bartegi et al, 1990). After addition of SDS sample buffer a 1D-SDS PAGE and a Western Blot with CtsR antibody were performed.

Error-prone PCR

An artificially constructed *clpC* operon with a *spec*^{R}-cassette (shown in Fig. 1a) was amplified from a plasmid using a standard error-prone PCR protocol (Eckert & Kunkel, 1991). The resulting DNA fragments were transformed into *B. subtilis* 168 carrying a *clpE`-bgaB* reporter construct at the *amyE* locus. This method allowed us to screen for inactive CtsR variants under 37°C on X-Gal containing LB plates. Blue colonies were isolated and their *ctsR* genes were sequenced.

Supplemental Figures:

Figure S1 Composition of the *clpC* operon in different phyla of Gram+ bacteria

Presence or absence of the two modulators of CtsR activity in the *clpC* operon among different Gram+ bacteria. McsB, which was identified in *B. subtilis* as crucial for the regulation of CtsR activity, is absent in some Gram+ phyla, which raises doubts about the current model. We conclude that CtsR activity is more likely regulated by a general and simpler mechanism in all Gram+ bacteria.

Figure S2 Transcriptional analysis of *clpE* in a *ywlE* mutant

Northern Blot analysis of *B. subtilis clpE* mRNA in the wild-type and a *ywlE* mutant strain during exponential growth (37°C) and heat stress (50°C).

Figure S3 CtsR is inactivated in a temperature dependent manner

(A-B) Binding of CtsR to the *clpE* promoter fragment which contains additional CtsR binding sites. EMSA analysis performed at 37°C (K_d=0.83 ±0.009 (A) or 50°C (K_d=11.34±0.09) (B) and quantification of CtsR binding to the *clpE* promoter at different temperatures (C).

Figure S4 Cross-linking of CtsR at different temperatures

CtsR was cross-linked with DNA (*clpC* promoter fragment) at 37°C and at 50°C to evaluate whether CtsR dimerization is influenced by the dissociation of CtsR from the DNA. The results show that the dissociation from the DNA takes place under the maintenance of the CtsR dimers.

Figure S5 Hot-spot mutations in CtsR

The *B. subtilis* CtsR protein sequence is depicted with several amino acids colored in red, where error-prone mutations led to an inactive CtsR protein. The Helix-turn-Helix motive and the winged HTH region are underlined.

Figure S6 Impact of different CtsR point mutations on CtsR dependent transcription

Northern Blot analysis of *B. subtilis clpE* mRNA in a $CtsR^{V16M}$ or $CtsR^{G65S}$ strain during exponential growth (37°C) and heat stress (50°C).

Figure S7 Modeled structure of mutant and wild-type CtsR

A theoretical structure of the winged HTH domain was modeled with the G64P substitution (red) using SWISS-MODEL (Arnold et al, 2006) and then compared with the CtsR crystal structure (Fuhrmann et al, 2009) (green). Areas where both structures are identical appear yellow. The G65P amino acid substitution shows no influence on the overall CtsR structure, except at position 64.

Figure S8 $CtsR^{G64P}$ is not temperature dependently inactivated

CtsR^{G64P} is not inactivated upon heat exposure and binds DNA with an equal intensity like under control conditions.

Figure S9 A kinase deficient McsB mutant is no longer able to bind CtsR during heat stress

Co-immunoprecipitation of McsB or McsB^{C167S} with CtsR during control or 10 minutes after heat stress followed by Western-Blot analysis with McsB and CtsR antibodies.

Figure S10 Degradation of CtsR in *L. lactis*

Pulse-chase labeling and immunoprecipitation of CtsR in *L. lactis* wild-type or *clpE* mutant cells during a temperature shift to 42°C.

Figure S11 Inactivation of CtsR during disulfide stress depends on the presence of McsB

Northern Blot analysis of *B. subtilis clpE* mRNA in the wild-type and a *mcsB* mutant strain during exponential growth and disulfide stress (1mM diamide).

Table S1. Strains used in this study.

strain/plasmid	Genotype or description	reference/construction
E. coli		
BL21(DE3)pLysS	F$^-$ *lon hsdS*$_B$ (r_B^- m_B^-) with DE3, a λ prophage carrying T7 RNA polymerase gene and plasmid pLysS containing T7 phage lysozyme gene	
B. subtilis		
168	*trpC2*	(Anagnostopoulos & Spizizen, 1961)
BMM1	*trpC2 amyE::cat (clpE`-bgaB)*	(Miethke et al, 2006)
BEK89	*trpC2 ΔmcsB::aphA3*	(Krüger et al, 2001)
BAE048	*trpC2 ΔywlE::ahpA3*	this study
BAE019	*trpC2 ctsR::spec^R*	this study
BAE051	*trpC2 ctsR::spec^R mcsB* ^{C167S}	this study

strain/plasmid	Genotype or description	reference/construction
BAE049	*trpC2* Δ*ywlE::ahpA3* Δ*clpC::tet*^{R}	this study
BAE056	*trpC2 ctsR::spec*^{R} *mcsB* ^{C167S} Δ*ywlE::ahpA3*	this study
BAE064	*trpC2 ctsR::spec*^{R} *ctsR* ^{G64P}	this study
BAE081	*trpC2* Δ*mcsB::aphA3 ctsR::spec*^{R} *ctsR* ^{G64P}	BAE064-->BEK89
BAE070	*trpC2 ctsR::spec*^{R} *ctsR* ^{R63K}	this study
BAE071	*trpC2 ctsR::spec*^{R} *ctsR* $^{R63K/R70K}$	this study
BAE072	*trpC2 ctsR::spec*^{R} *ctsR* $^{R28K/R50K/R55K/R63K/R70K}$	this study
BAE085	*trpC2 amyE::cat* (*clpE`-bgaB*) Δ*ctsR::aphA3* pDG148 *::ctsR-G.stearothermophilus*	this study
BAE033	*trpC2 ctsR::spec*^{R} *ctsR* ^{V16M}	this study
BAE034	*trpC2 ctsR::spec*^{R} *ctsR* ^{G65S}	this study
BDZ12	*trpC2* amyE::cat (clpE`-bgaB) Δ*ctsR::aphA3* pDG148 *::ctsR-L.lactis*	this study
L. lactis		
MG1363	Wild-type *L. lactis* subsp. *cremoris*	
HI1931	MG1363 Δ*clpE::tet*^{R}	(Varmanen et al, 2003)
plasmids		
pDG148	*lacI*/pspac/*kan*/*ble*/*B. subtilis ori* pUB110/*E. coli ori* pBR322/Amp^R	(Stragier et al, 1988)
pRSETA	cloning vector, Amp^R	invitrogen
pRSETA-CtsR	cloning vector, Amp^R	(Krüger & Hecker, 1998)
pRSETA-CtsR-*L.lactis*	cloning vector, Amp^R	this study
pRSETA-CtsR-*L.lactis*-G65P	cloning vector, Amp^R	this study
pRSETA-CtsR-*G. stearothermophilus*	cloning vector, Amp^R	this study
pRSETA-CtsR-*G. stearothermophilus*-G64P	cloning vector, Amp^R	this study
pRSETA-CtsR-G64P	cloning vector, Amp^R	this study

strain/plasmid	Genotype or description	reference/construction
pUS19-*clpC*strep	integration vector for *B. subtilis*	this study
pDG148-CtsR-*L.lactis*	*lacI*/pspac/*kan*/*ble*/*B. subtilis ori* pUB110/*E. coli ori* pBR322/Amp^R	this study
pDG148-CtsR-*G. stearothermophilus*	*lacI*/pspac/*kan*/*ble*/*B. subtilis ori* pUB110/*E. coli ori* pBR322/Amp^R	this study

aphA3, *cat*, *tet* and *spec* stand for resistance to kanamycin, chloramphenicol, tetracyclin and spectinomycin, respectively.

Table S2. Primer used in this study.

purpose	name	sequence (5´-3´)
in vivo genetic system	McsA_Bfor2_up	GTGGGACATAATATTTCTGAC
	McsA_Brev_down	TTCCTAGAAGCTGGAGCACC
	CtsRrev_spec_SD_up	CAAATATATCCTCCTCACTATTTATTTTAATTTTAAAGAAGTCAGC
	McsA_SD_spec_OT	AAATAACAGATTAAAAAAATTATAATAAGCGGGTGAAAAGATTGATTTGTCAAG
	Spec_for_SD	ATAGTGAGGAGGATATATTTG
	Spec_rev_OT	TTATAATTTTTTTAATCTGTTATTT
Δ*ywlE*	YwlE_up_for	GTCGGCCTTGGAATGGGCATG
	YwlE_down_rev	CATTATGCACCCTCGTCGC
	YwlE_km_rev	GTGATATTCTCATTTTAGCCATGTCAGTCACCCCTTATTTTTC
	km_for	GAAAAATAAGGGGTGACTGACATGGCTAAAATGAGAATATCAC
	Ywle_km_for	TTACTGGATGAATTGTTTTAGGTTGTCAGAAAATCTGCAAAC
	Km_rev	GTTTGCAGATTTTCTGACAACCTAAAACAATTCATCCAGTAA
substitution mutants	McsB_GTS_C167S_for	CAAAGAGGATACTTAACCAGCTCTCCTACAAACGTA
	McsB_GTS_C167S_rev	GCTGGTTAAGTATCCTCTTTGCTCATTGAATG
	CtsR_G64P_GTS_for	ATTGTTGAGAGCAAACGCGGGCCCGGCGGTTACA
	CtsR_G64P_GTS_rev	CCCGCGTTTGCTCTCAACAATATATCCTCTTTC

purpose	name	sequence (5´-3´)
	MCSB_STREP_FOR	ATGGCTAGCTGGAGCCACCCGCAGTTCGAAAAAGGCGCCATGTCGCTAAAGCATTTTATTCAGG
	MCSB_STRET_REV	GGCGCCTTTTTCGAACTGCGGGTGGCTCCAGCTAGCCATTACTCCTGTTCCTCCTCACTATC
	clpC-STREP-for	CGCGGATCCCAATTGACAGAGAAAGTCAGAAGAAA
	clpC-STREP-rev	CGGAATTCTTATTTTTCGAATTGCGGATGAGACCACGCAGAATTCGTTTTAGCAGTCGTTTTTAC
	R63L_GTS_for	GATATATTGTTGAGAGCAAAAAAGGGGGCGGCGGT
	R63L_GTS_rev	TTTGCTCTCAACAATATATCCTCTTTCGCT
	CtsR_GTS_R70L_for	CGGGGGCGGCGGTTACATCAAAATCATTAAAAT
	Ctsr_GTS_R70rev	GATGTAACCGCCGCCCCCGCGTTTGCTCTC
	CtsR_R28L_for	AGGAAATTTTAGAGATTAAAAAAAGTGAAATTGCAG
	CtsR_R28L_rev	TTTAATCTCTAAAATTTCCTTGCCATTTTG
	CtsR_R5055L_for	TAAATTATGTCATCAACACCAAATTTACAAGCGAAAAAGGATATATTG
	CtsR_R5055L_rev	GGTGTTGATGACATAATTTATTTGGGAAGGAAC
ectopic expression of different CtsR's from pDG148	pDG_CtsR_bt_sall	ACGCGTCGACGTGCCGAACATTTCCGACAT
	pDG_CtsR_bt_sphI	CCCGCATGCCTATTTGTATTTCAGCGACGT
	ctsr_llac_for	GTCGACATGGCAGGTCAAAAAAATAC
	ctsr_llac_rev	GCATGCTCAAATTTCATCGTCACGGTC
over-expression of different CtsR's from pRSETA	prsetA_CtsR_bt_Bam	GGAGGATCCGTGCCGAACATTTCCGACAT
	prsetA_CtsR_bt_Eco	GAAGAATTCCTATTTGTATTTCAGCGACGT
	pRSETA_CtsR_Ll_Bam	GGAGGATCCATGGCAGGTCAAAAAAATACAT
	pRSETA_CtsR_Ll_kpNI	GGTGGTACCTCAAATTTCATCGTCACGGTC

References and Notes

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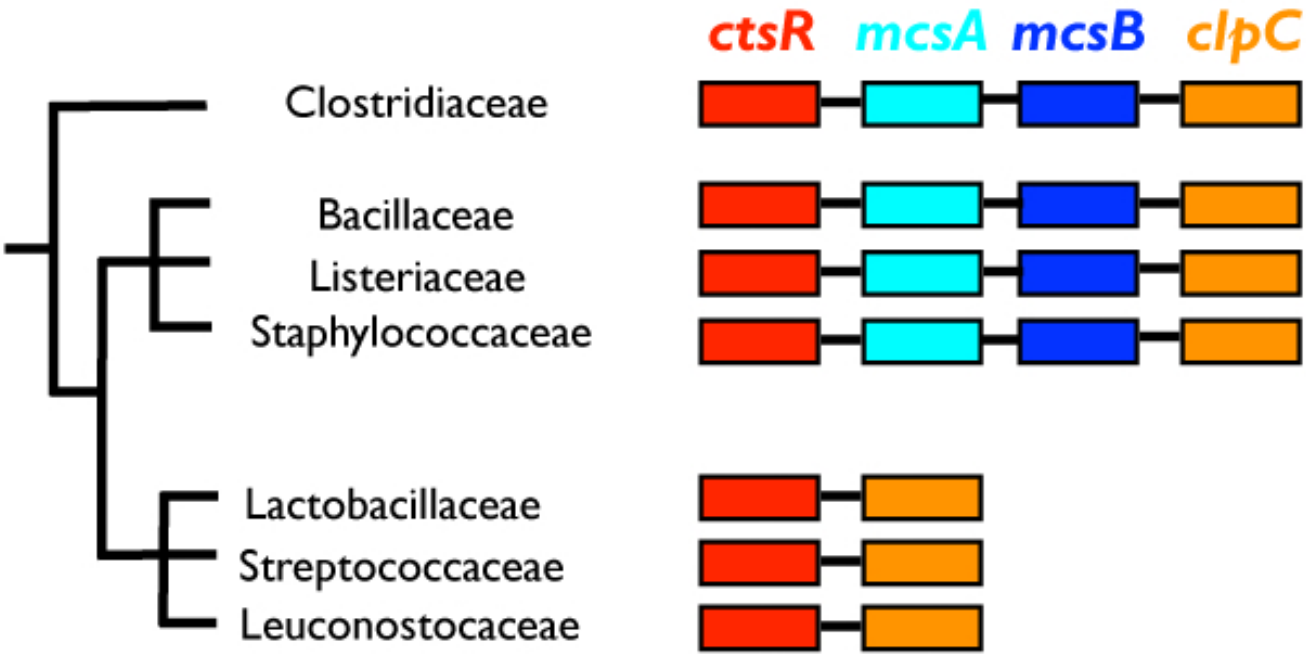
Figure S1:


ctsR
mcsA
mcsB
clpC
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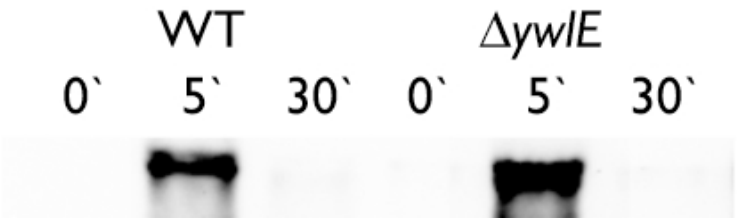
Streptococcaceae
Leuconostocaceae

Figure S2:



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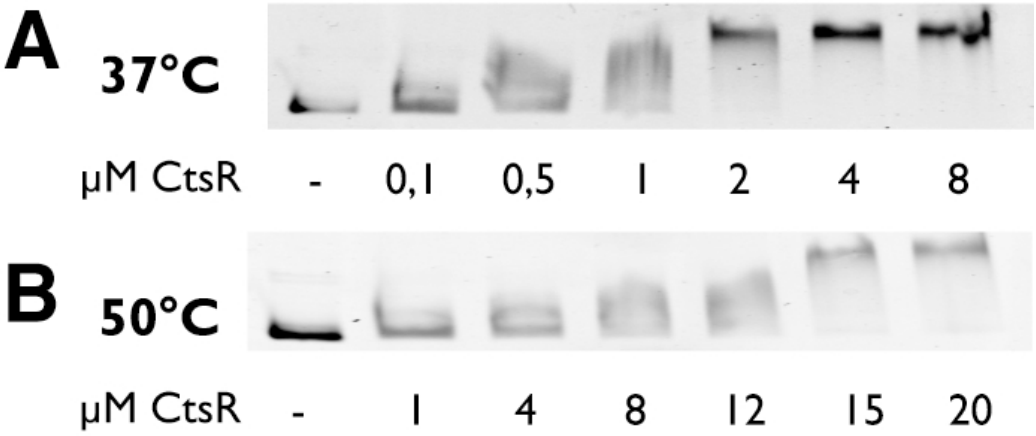
A
37°C
µM CtsR - 0,1 0,5 1 2 4 8
B
50°C
µM CtsR - 1 4 8 12 15 20

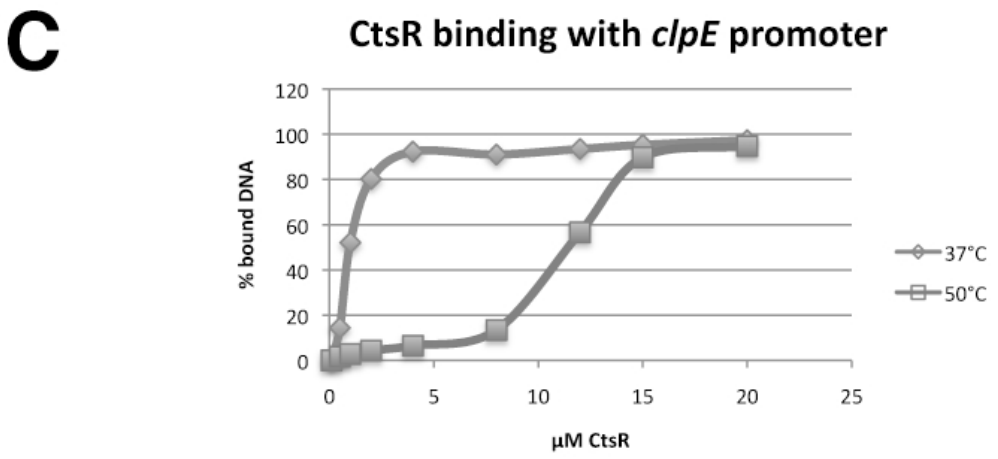
C
CtsR binding with clpE promoter
% bound DNA
120
100
80
60
40
20
0
0
5
10
15
20
25
µM CtsR
37°C
50°C

Figure S4:

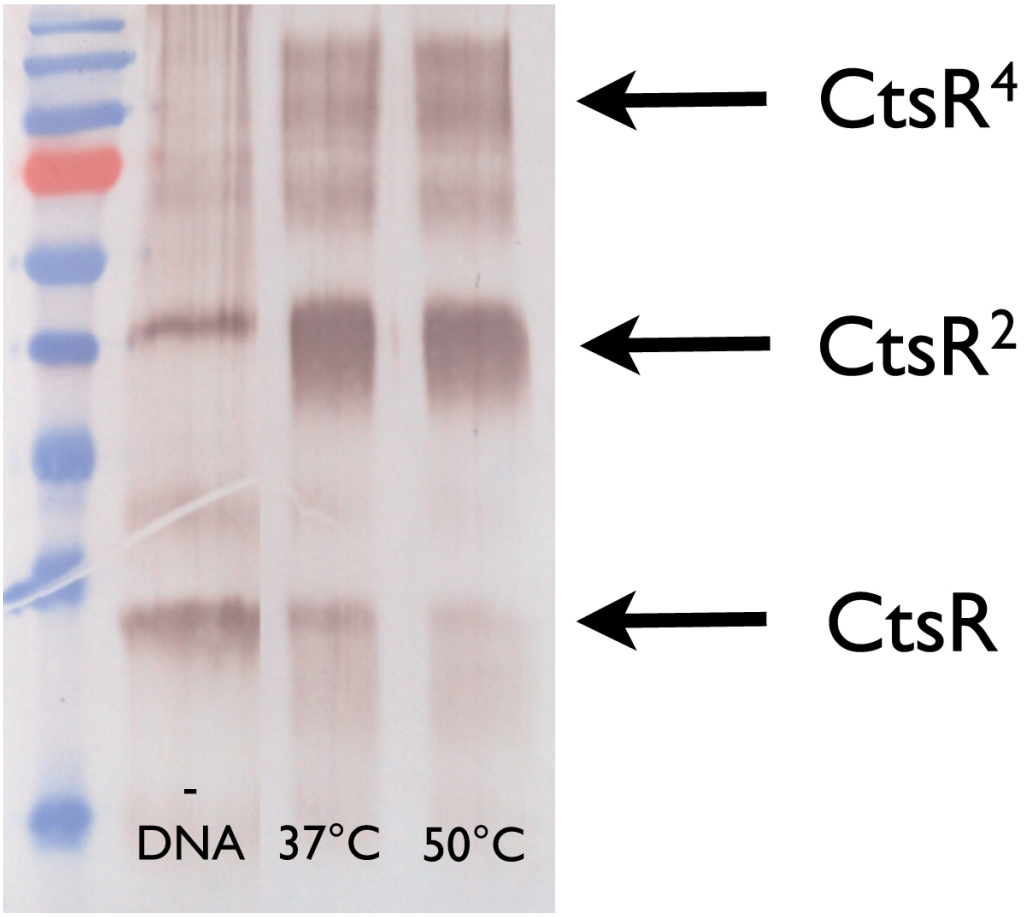

Figure S5:


1 - MGHNISDIIE QYLKRVLDQN GKEILEIKRS EIADKFQCVP SQINYVINTR FTSERGYIVE
Helix
TURN
Helix
61 - SKRGGGGYIR IIKIKMNNEV VLINNIISQI NTHLSQAASD DIILRLLEDK VISEREAKMM
winged HTH
121 - VSVMDRSVLH IDLPERDELR ARMMKAMLTS LKLK

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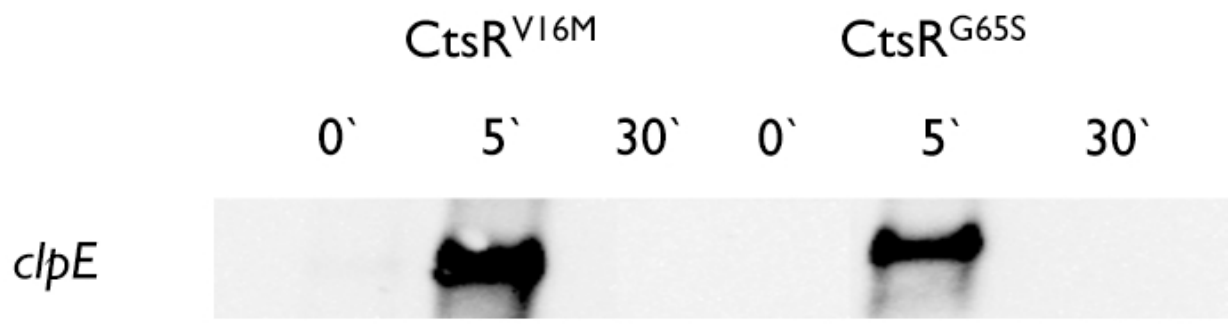

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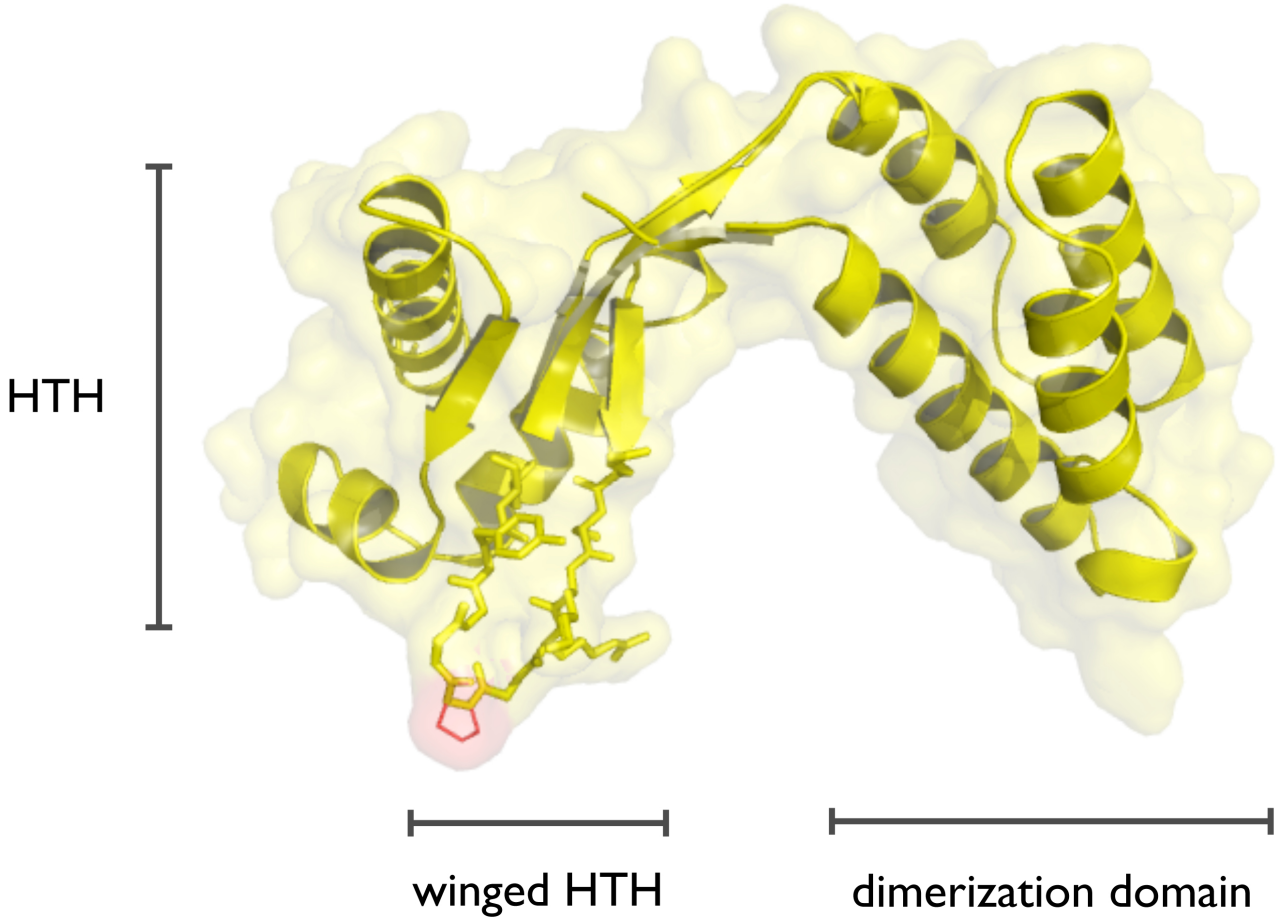

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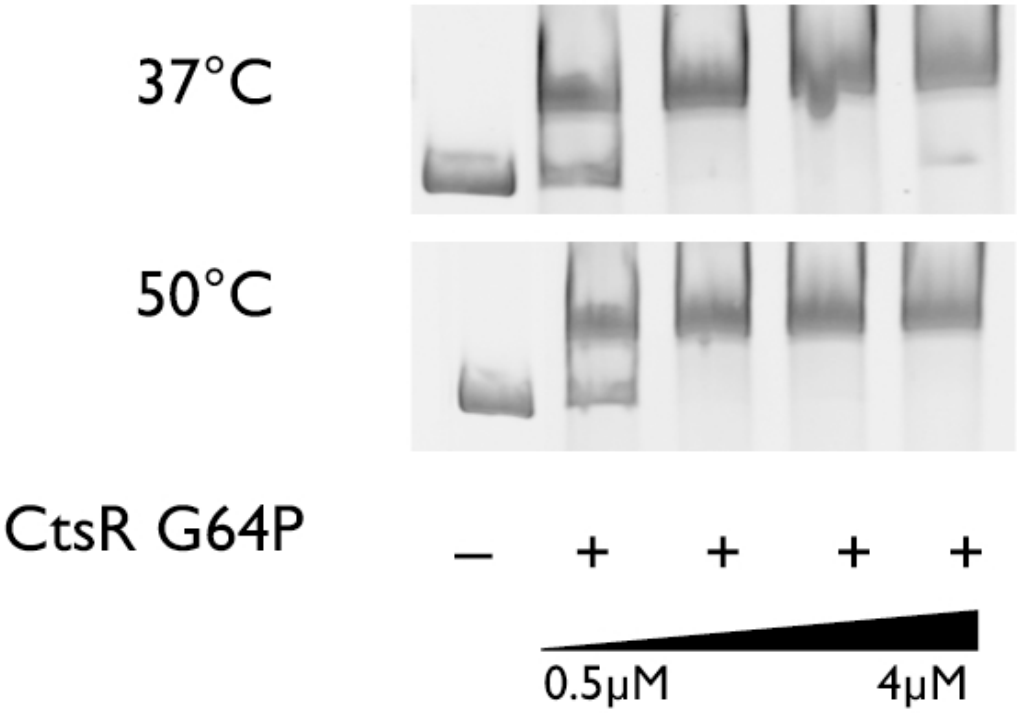

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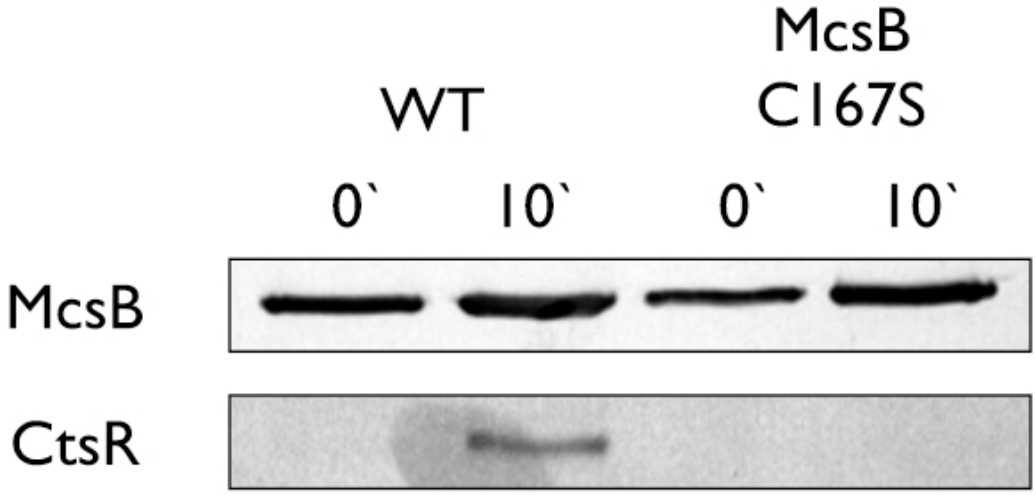

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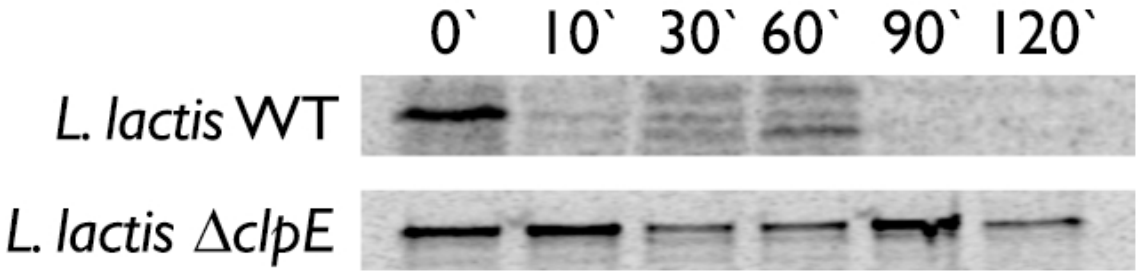

Figure S11: