Supplementary Material

Distinct contributions of O-acetylserine sulfhydrylases to cysteine biosynthesis in *Pseudomonas aeruginosa*

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SUPPLEMENTARY METHODS

Rapid-scanning stopped-flow experiments

Rapid-scanning stopped-flow experiments were carried out using a DX.17MV spectrophotometer (Applied Photophysics, Leatherhead, UK) equipped with a diode-array detector. The enzymes (32 μM) were rapidly mixed with 1 mM OAS. Spectra were collected in the 300 - 600 nm range at the following time point after mixing 12, 24, 26, 48, 60, 192, 204, 216, 228, 240 ms. Spectra at time zero (t_0) correspond to the enzyme in the absence of substrate. All experiments were conducted in 20 mM sodium phosphate buffer, pH 8.0, at 15 °C. Differential spectra ($\Delta A = A_{tx} - A_{to}$) were calculated, where A_{to} represents the spectrum prior to substrate addition and A_{tx} spectra recorded at subsequent reaction times.

SUPPLEMENTARY TABLES

Table S1. Steady-state kinetic parameters of PaCysK and PaCysM at 25 °C using OAS and TNB as substrates. Values represent mean ± SEM from ≥ 3 independent measurements, obtained using at least two different protein batches.

	PaCysK	**PaCysM**	**StCysK***	**StCysM***
$\mathbf{k_{cat}}$ (s^{-1})	0.02 ± 0.01	1.5 ± 0.2	0.56 ± 0.08	8 ± 2
$\mathbf{K_m}$ **(OAS)** (mM)	0.50 ± 0.03	0.09 ± 0.03	15 ± 3	0.19 ± 0.08
$\mathbf{K_m}$ **(TNB)** (mM)	0.04 ± 0.02	0.06 ± 0.02	0.6 ± 0.1	0.43 ± 0.09
$\boldsymbol{k_{cat}/K_m}$ **(OAS)** (M^{-1} s^{-1})	40 ± 20	(1.7 ± 0.6) × 10^4	37 ± 5	(4.1 ± 1.9) ×10^4
$\boldsymbol{k_{cat}/K_m}$ **(TNB)** (M^{-1} s^{-1})	500 ± 350	(2.5 ± 0.9) ×10^4	950 ± 55	(1.8 ± 0.1) ×10^4

*For comparison, kinetic parameters of *Salmonella enterica* serovar Typhimurium homologs (StCysK and StCysM) are reported [1].

Table S2. Bacterial strains used in this study

Strains	**Relevant characteristics**	**Reference/Source**
E. coli		
DH5α	Cloning strain	[S2]
S17.1λ*pir*	Conjugative strain for suicide plasmids	[S3]
P. aeruginosa		
PAO1	Reference isolate, wild type, ATCC 15692 type strain	American Type Culture Collection
Δ*cysK*	PAO1 derivative strain carrying a deletion of the *cysK* gene (PA2709), obtained by allelic exchange using the plasmid pDM4Δ*cysK* (**Table S2**).	This study
Δ*cysM*	PAO1 derivative strain carrying a deletion of the *cysM* gene (PA0932), obtained by allelic exchange using the plasmid pDM4Δ*cysM* (**Table S2**).	This study
Δ*cysKM*	Δ*cysM* derivative strain carrying a deletion of the *cysK* gene (PA2709), obtained by allelic exchange using the plasmid pDM4Δ*cysK* (**Table S2**).	This study

Table S3. Plasmids used in this study.

Plasmids	Relevant characteristics and plasmids construction	Reference/Source
pDM4	Suicide vector; *sacBR*; *oriR6K*; Cm^R.	[S4]
pME6032	IPTG inducible expression vector, *lacI*^{q}-P_{tac}, Tc^R.	[S5]
pDM4Δ*cysK*	pDM4-derived plasmid for the generation of the Δ*cysK* mutant strain; Cm^R. It contains the DNA fragments encompassing the upstream region of the *cysK* gene originated with primers FW*cysK*UP and RV*cysK*UP (**Table S3**), and the downstream region of the *cysK* gene originated with primers FW*cysK*DW and RV*cysK*DW (**Table S3**), cloned in pDM4 by XhoI-XbaI restriction.	This study
pDM4Δ*cysM*	pDM4-derived plasmid for the generation of the Δ*cysM* mutant strain; Cm^R. It contains the DNA fragments encompassing the upstream region of the *cysM* gene originated with primers FW*cysM*UP and RV*cysM*UP (**Table S3**), and the downstream region of the *cysM* gene originated with primers FW*cysM*DW and RV*cysM*DW (**Table S3**), cloned in pDM4 by XhoI-XbaI restriction.	This study
pME-*cysK*	pME6032 derivative carrying the coding sequence of *cysK* downstream of the IPTG-inducible P_{tac} promoter. The *cysK* gene was amplified from PAO1 genome with the primer pair *cysK*-FW and *cysK*-RV (**Table S3**), and cloned in pME6032 by EcoRI-KpnI restriction.	This study
pME-*cysM*	pME6032 derivative carrying the coding sequence of *cysM* downstream of the IPTG-inducible P_{tac} promoter. The *cysM* gene was amplified from PAO1 genome with the primer pair *cysM*-FW and *cysM*-RV (**Table S3**), and cloned in pME6032 by EcoRI-KpnI restriction.	This study

Table S4. Oligonucleotides used in this study.

Name	Sequence (5'-3')[a]	Restriction site
FW*cysK*UP	CCGCTCGAGGAGCTGCGGACGCTCGAA	XhoI
RV*cysK*UP	CCGGAATTCCTTGGCCAGGATGGTGACG	EcoRI
FW*cysk*DW	CCGGAATTCACGGCCTGTTCAGCGAACA	EcoRI
RV*cysK*DW	GCTCTAGAGAGCTTCTCGGCGAAGCT	XbaI
FW*cysM*UP	CCGCTCGAGGGCCGCCAGGAGCCGC	XhoI
RV*cysM*UP	CCCAAGCTTAGGGGTATTGCCAACGCAG	HindIII
FW*cysM*UDW	CCCAAGCTTCCTGTCTTCCGGCGTCTAT	HindIII
RV*cysM*DW	GCTCTAGAACGGAAGCCGACATCCAGA	XbaI
cysK-FW	CCGGAATTCATGAGCCGCATCTTCGC	EcoRI
cysK-RV	CGGGGTACCTTACTGGGTCAGTTCCTGTT	KpnI
cysM-FW	CCGGAATTCATGACCGTGCAGTACCC	EcoRI
cysM-RV	CGGGGTACCTCAGCGCGGGTCATAGA	KpnI

[a] Restriction sites are underlined in the primer sequences.

SUPPLEMENTARY FIGURES

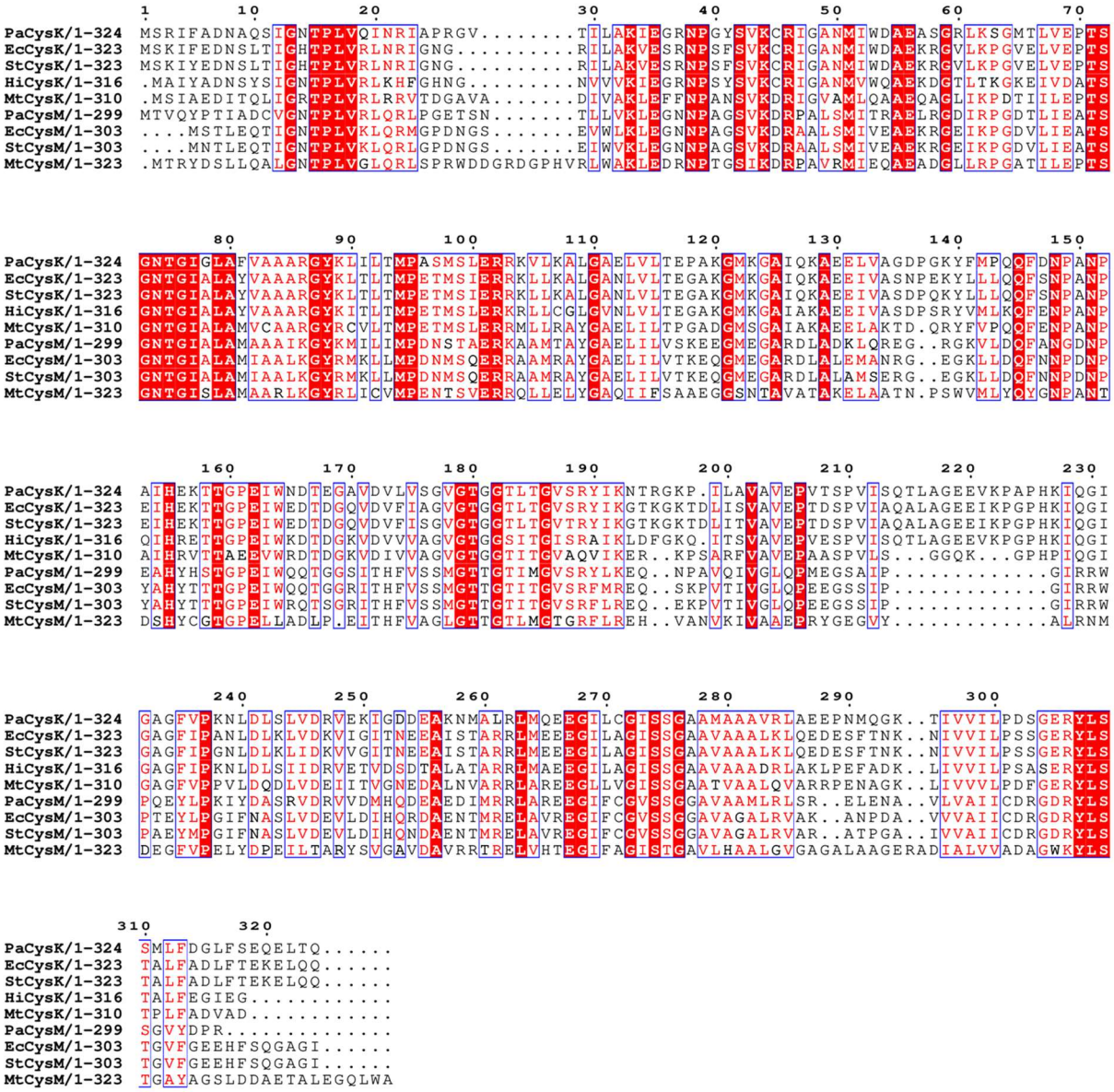

Figure S1. Multiple sequence alignment of CysK and CysM. *P. aeruginosa* CysK (PaCysK, UniProt ID: Q9I0D3), *E. coli* CysK (EcCysK, UniProt ID: P0ABK5), *Salmonella enterica* serovar Typhimurium CysK (StCysK, UniProt ID: P0A1E3), *H. influenzae* CysK (HiCysK, UniProt ID: P45040), *M. tuberculosis* CysK1 (MtCysK1, UniProt ID: P9WP55), *P. aeruginosa* CysM (PaCysM, UniProt ID: Q9I526), *E. coli* CysM (EcCysM, Uniprot ID: P16703), *S. enterica* ser. Typhimurium CysM (StCysM, UniProt ID: P29848), *M. tuberculosis* CysM (MtCysM, UniProt ID: P9WP53).

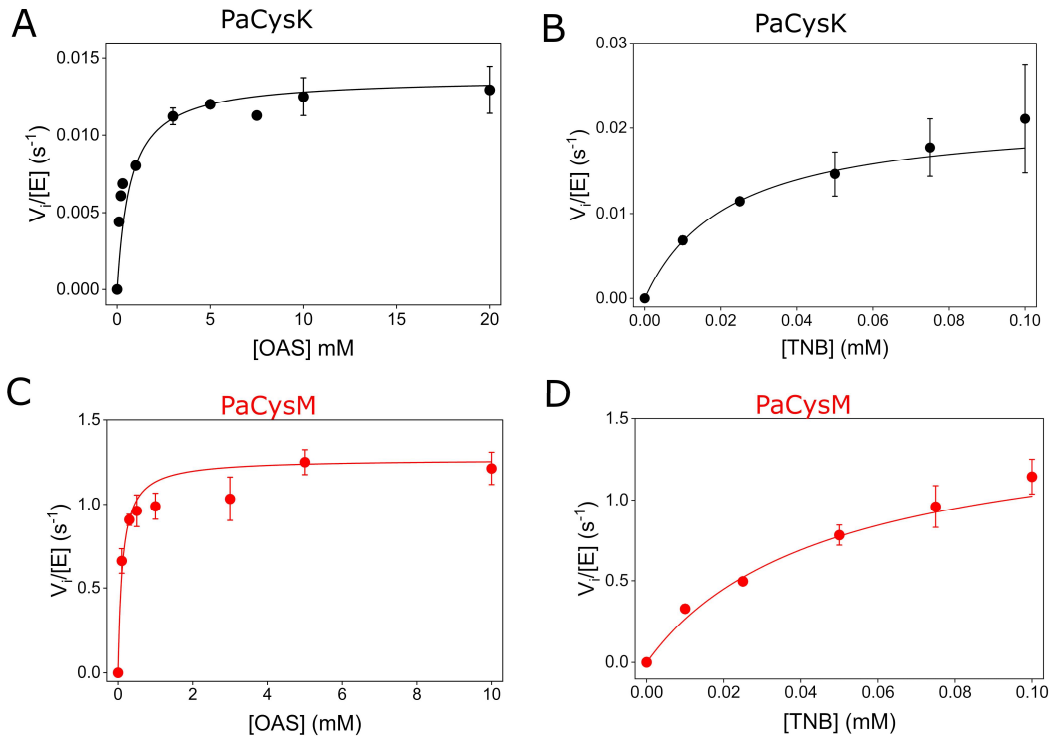

Figure S2. Steady-state enzyme kinetics of PaCysK and PaCysM using OAS and TNB as substrates. (A-B) PaCysK reaction rates as a function of OAS concentration (**A**) and TNB concentration (**B**). (**C-D**) PaCysM reaction rates as a function of OAS concentration (**C**) and TNB concentration (**D**). Kinetic parameters derived from these experiments are summarized in **Table S1**.

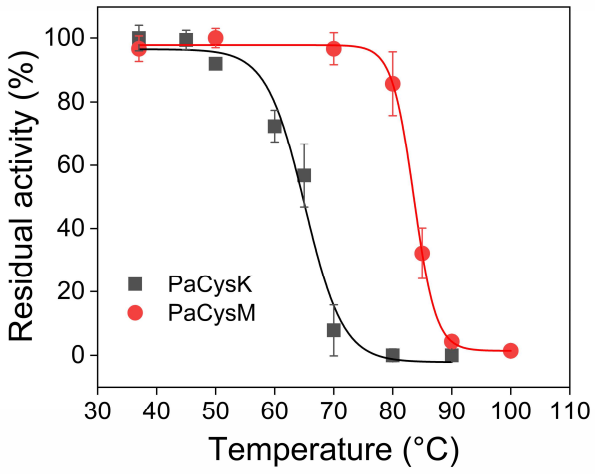

Figure S3. Residual activity of PaCysK and PaCysM at increasing temperatures. Activity was measured using 10 mM OAS with 0.5 mM Na_2S for PaCysK, and 5 mM Na_2S for PaCysM, after incubating the enzymes for 10 minutes at various temperatures.

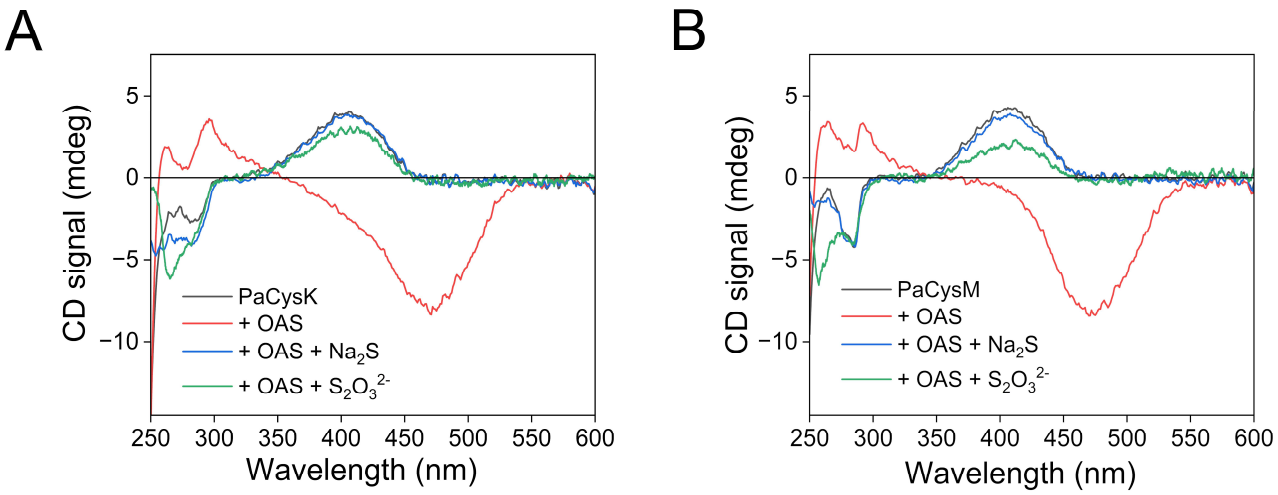

Figure S4. Near-UV visible CD spectra of PaCysK and PaCysM. CD Spectra of 1 mg mL^{-1} PaCysK (A) and PaCysM (B) alone (black), after addition of OAS (red), and after subsequent addition of Na_2S (blue) or $Na_2S_2O_3$ (green) to the protein-OAS complex.

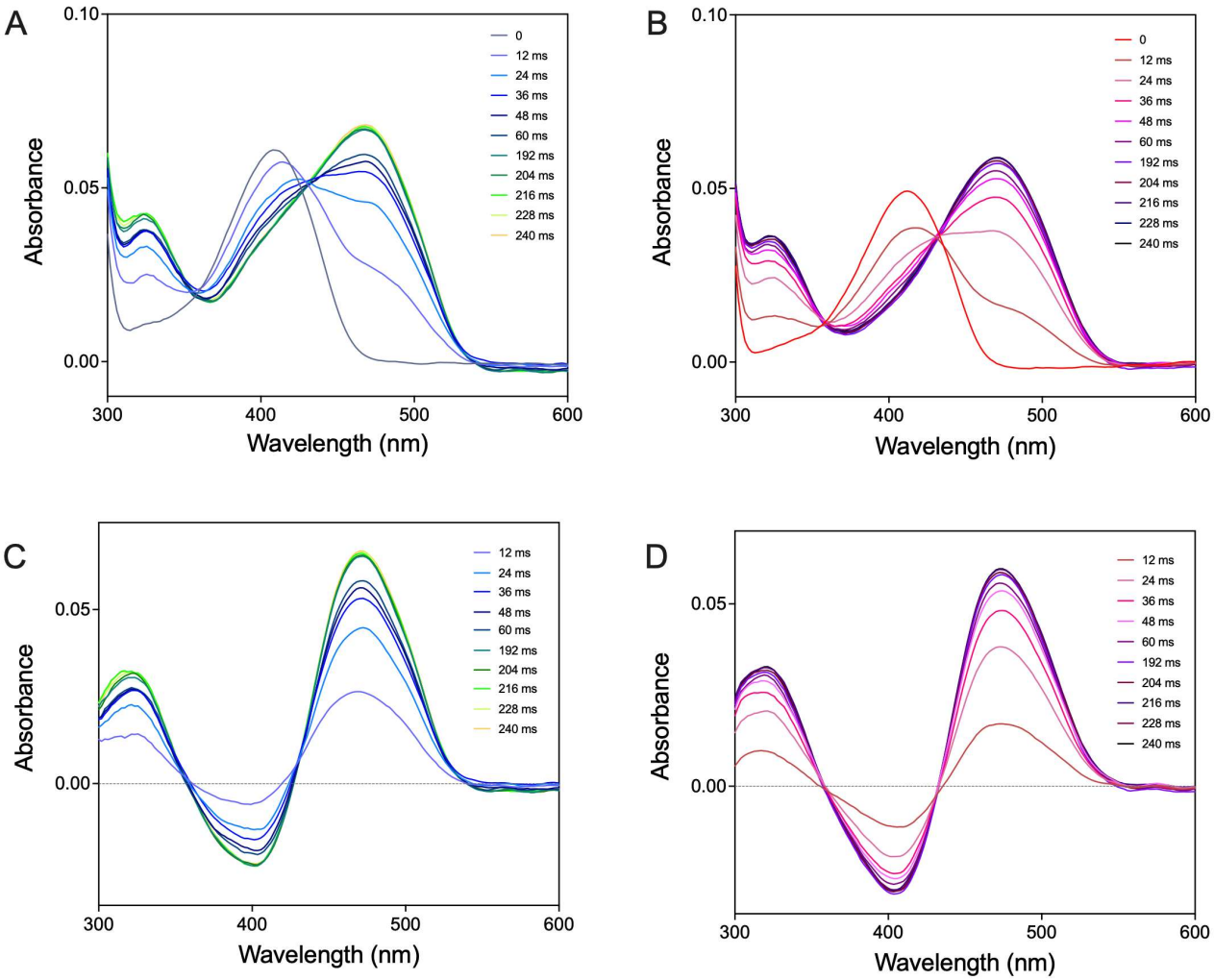

Figure S5. (**A, B**) Time-resolved absorption spectra acquired after stopped-flow mixing 32 µM PaCysK (**A**) or PaCysM **(B**) with 1 mM OAS. Spectrum t_0 corresponds to the enzyme before mixing, while subsequent spectra were recorded at the indicated times after mixing with OAS. (**C, D**) Corresponding difference spectra ($\Delta A = At_x - At_0$, where At_0 is the spectrum of the enzyme before mixing and At_x refers to the spectra collected at the indicated reaction times) for PaCysK (**C**) and PaCysM (**D**). The spectra highlight the absence of a single isosbestic point in the earliest time windows, indicating the transient formation of a short-lived intermediate during the first milliseconds of the reaction. In the spectra collected at 12 and 24 ms after mixing, the signals between 370 and 500 nm reflect a combination of the external Schiff base and the α-aminoacrylate species.

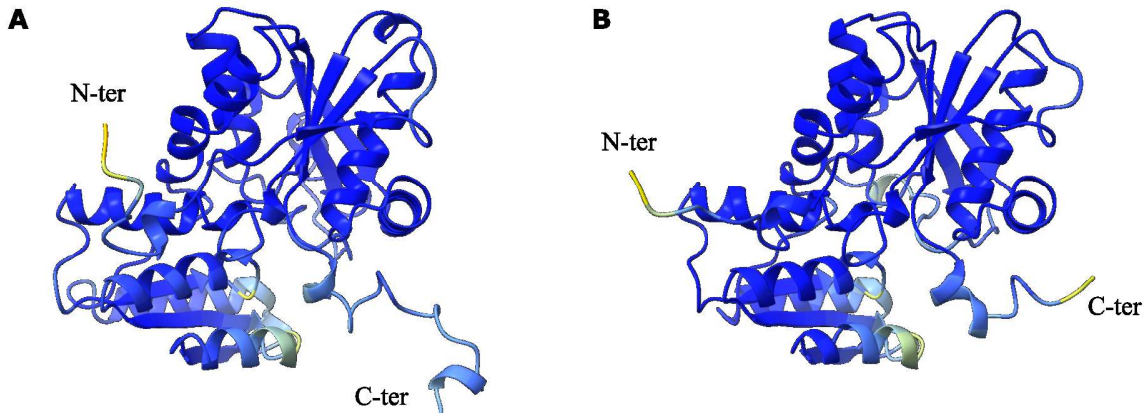

Figure S6. AlphaFold models of PaCysM and PaCysK. AlphaFold models of PaCysM (AF-Q9I0D3-F1) (A) and PaCysK (AF-Q9I526-F1) (B), colored according to the predicted Local Distance Difference Test (pLDDT) score.

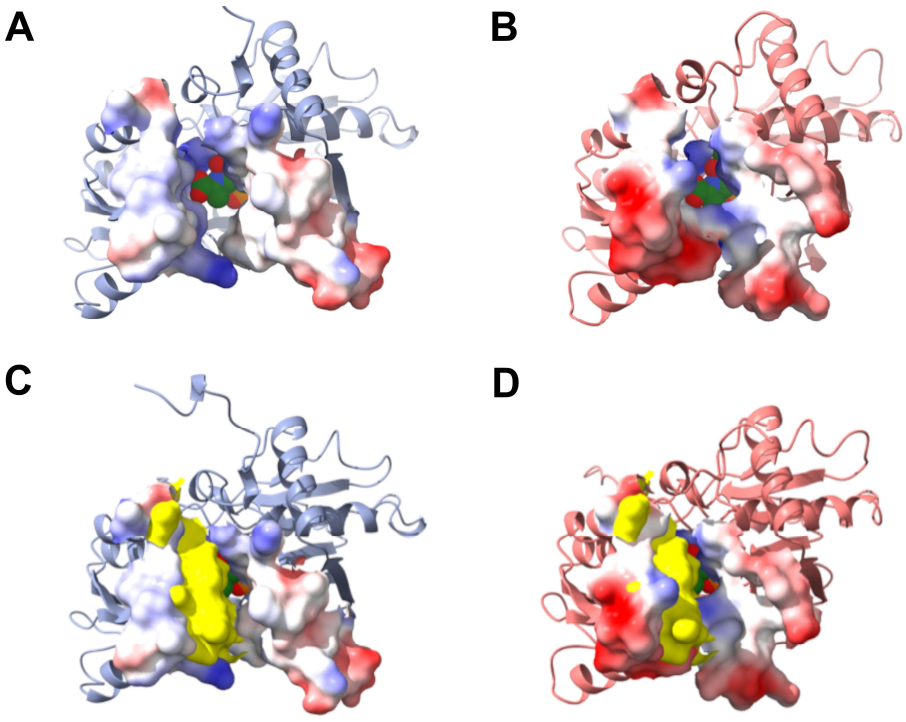

Figure S7. Electrostatic surface and structural comparison of the putative active sites of PaCysK and PaCysM with α-aminoacrylate intermediate. Electrostatic surface representation of the putative active site region of **(A)** AF-Q9I0D3-F1 (PaCysK, grey) and **(B)** AF-Q9I526-F1 (PaCysM, pink). The α-aminoacrylate intermediate, positioned based on structural alignment with the reference model of MtCysK (PDB: 2Q3D), is shown as spheres. On the right, the segment of MtCysK1 that folds over the active site upon interaction with the α-aminoacrylate has been superimposed onto the PaCysK **(C)** and PaCysM **(D)** models, suggesting a possible mode of active site closure during the formation of the complex with α-aminoacrylate in *P. aeruginosa*.

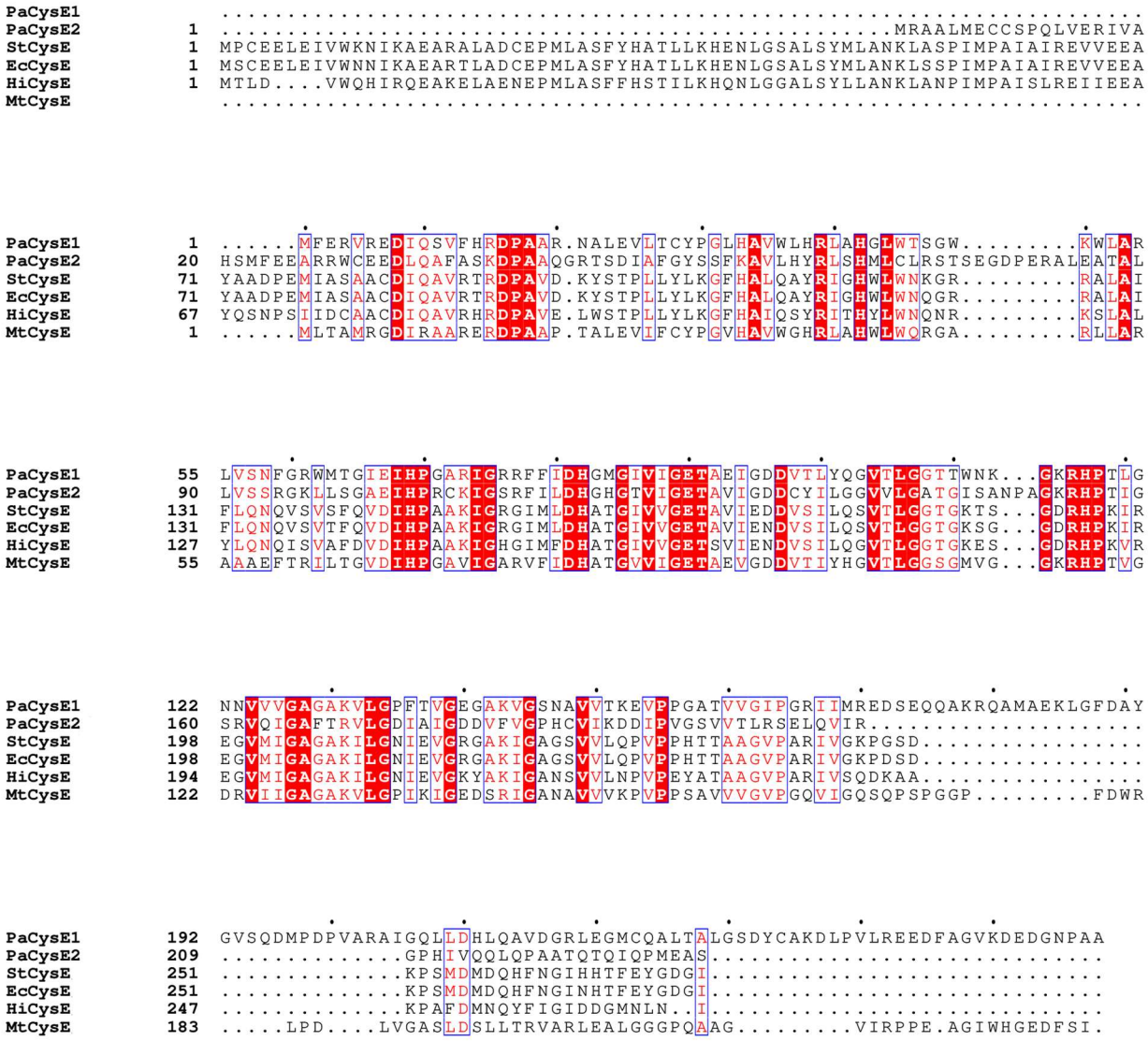

Figure S8. Multiple sequence alignment of CysE from different bacteria. *P. aeruginosa* CysE1 (PaCysE1, Uniprot ID: Q9HXI6), *P. aeruginosa* CysE2 (PaCysE2, Uniprot ID: Q9I210). *Salmonella enterica* serovar Typhimurium CysE (StCysE, Uniprot ID: P29847), *E. coli* CysE (EcCysE, Uniprot ID: P0A9D4), *H. influenzae* CysE (HiCysE, Uniprot ID: P43886), *M. tuberculosis* CysE (MtCysE, Uniprot ID: P95231).

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