MISSIONE 4
ISTRUZIONE
RICERCA

Finanziato dall'Unione europea
NextGenerationEU

Ministero dell'Università e della Ricerca

Italiadomani
PIANO NAZIONALE DI RIPRESA E RESILIENZA

De novo L-cysteine biosynthesis as a target for antibiotic development: identification of *Pseudomonas aeruginosa* CysH inhibitors *via* a multidisciplinary approach

VITALITY

Emanuela Frangipani, MSc, PhD

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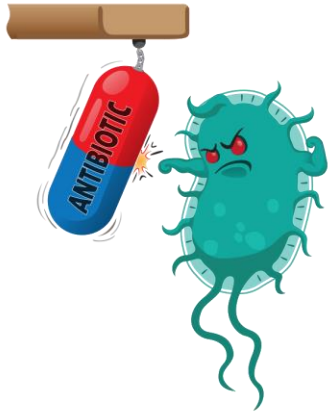
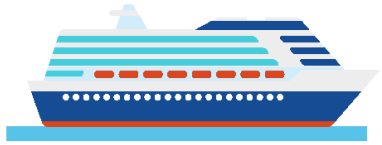


Antimicrobial resistance

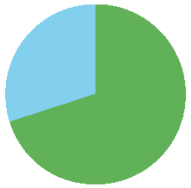
World Health Organization

More than
35000 deaths

Each year, more than 35 000 people die from antibiotic-resistant infections in the European Union, Iceland and Norway. This is **equivalent to the number of passengers on 13 cruise ships.**



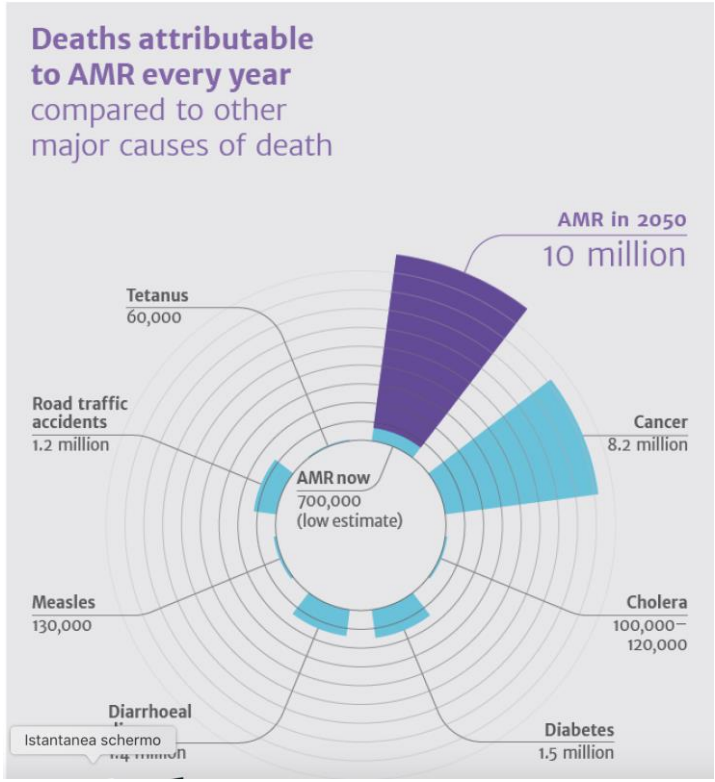
Antibiotic resistance is a silent pandemic and a growing threat to human health.



Over 70 %
healthcare-associated infections

Over 70% of the health impact of antibiotic-resistant infections is directly linked to healthcare-associated infections. This could be minimized through adequate infection prevention and control measures, as well as antibiotic stewardship in healthcare settings.

ecdc
EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL



CDC Centers for Disease Control and Prevention
CDC 24/7: Saving Lives, Protecting People™

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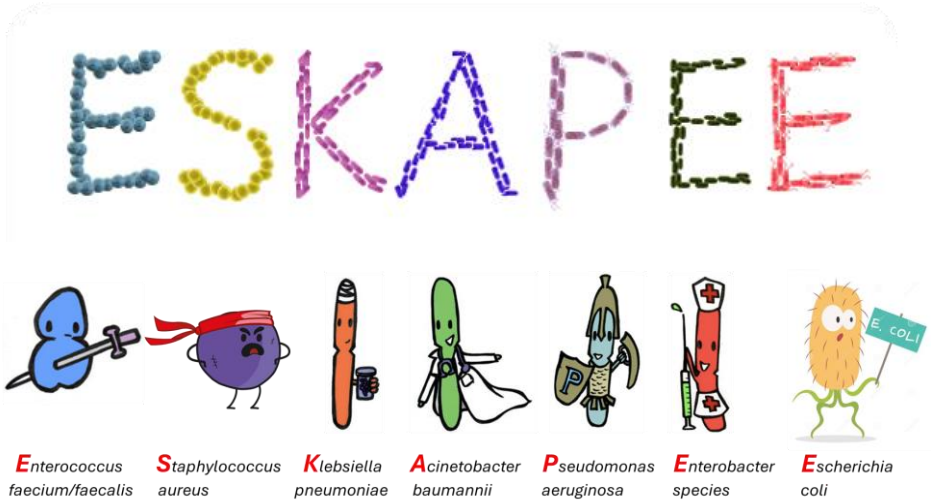
Antimicrobial resistance

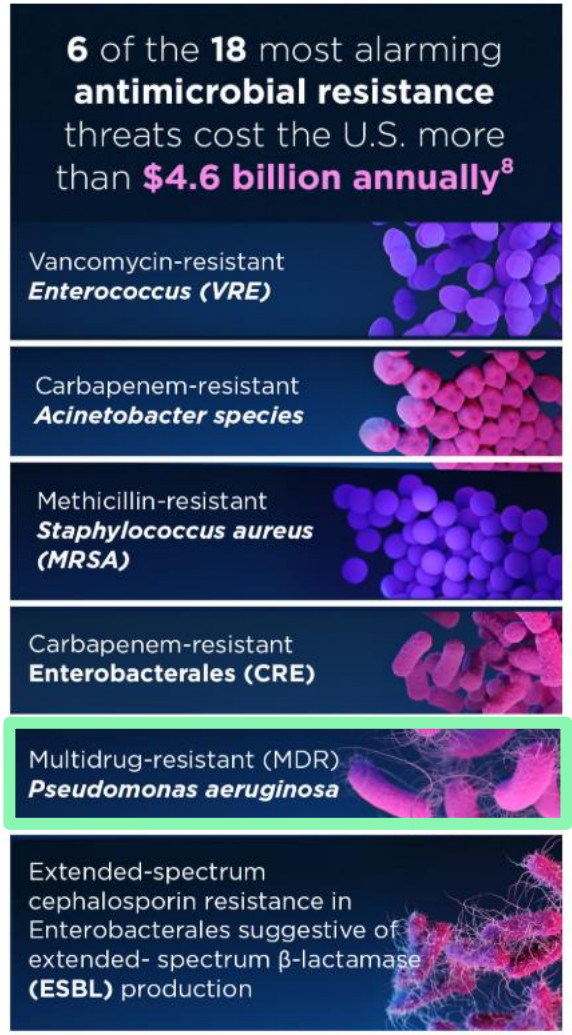
WHO Bacterial Priority Pathogens List, 2024

IDSA REPORT

Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America

Helen W. Boucher,[1] George H. Talbot,[2] John S. Bradley,[3,4] John E. Edwards, Jr,[5,6,7] David Gilbert,[8] Louis B. Rice,[9,10] Michael Scheld,[11] Brad Spellberg,[5,6,7] and John Bartlett[12]





U.S. Impact on Antimicrobial Resistance, Special Report 2022

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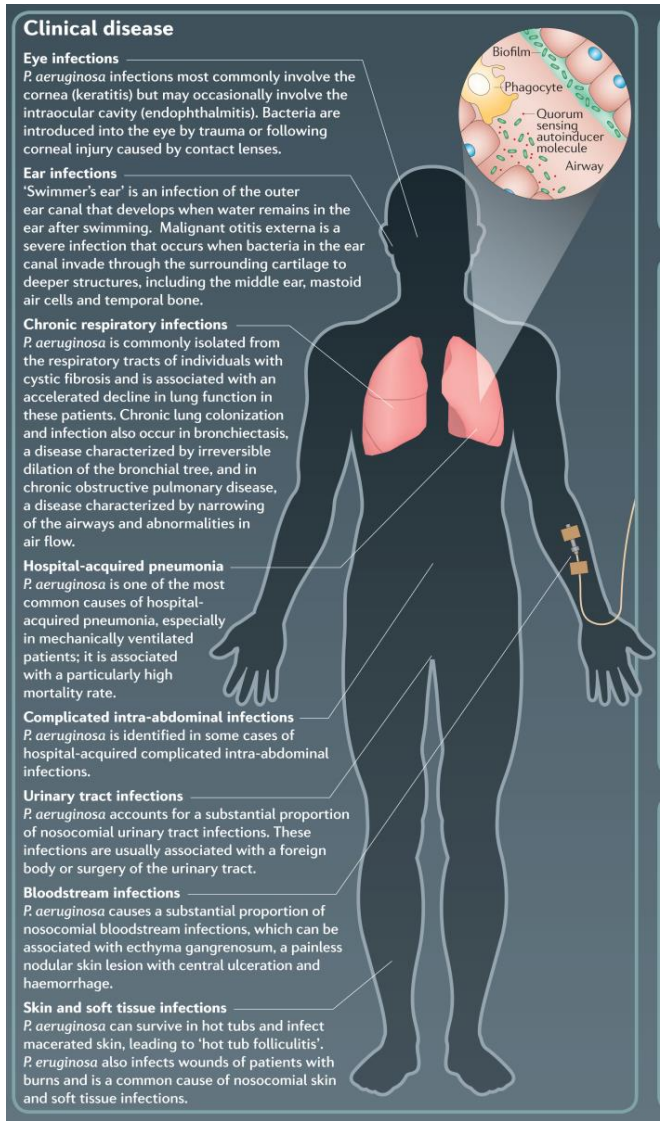
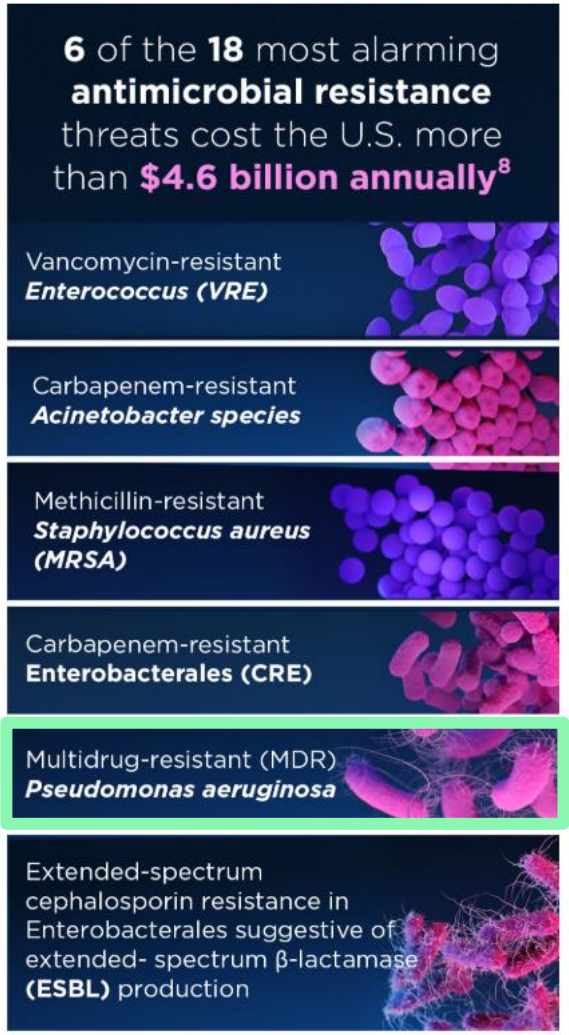
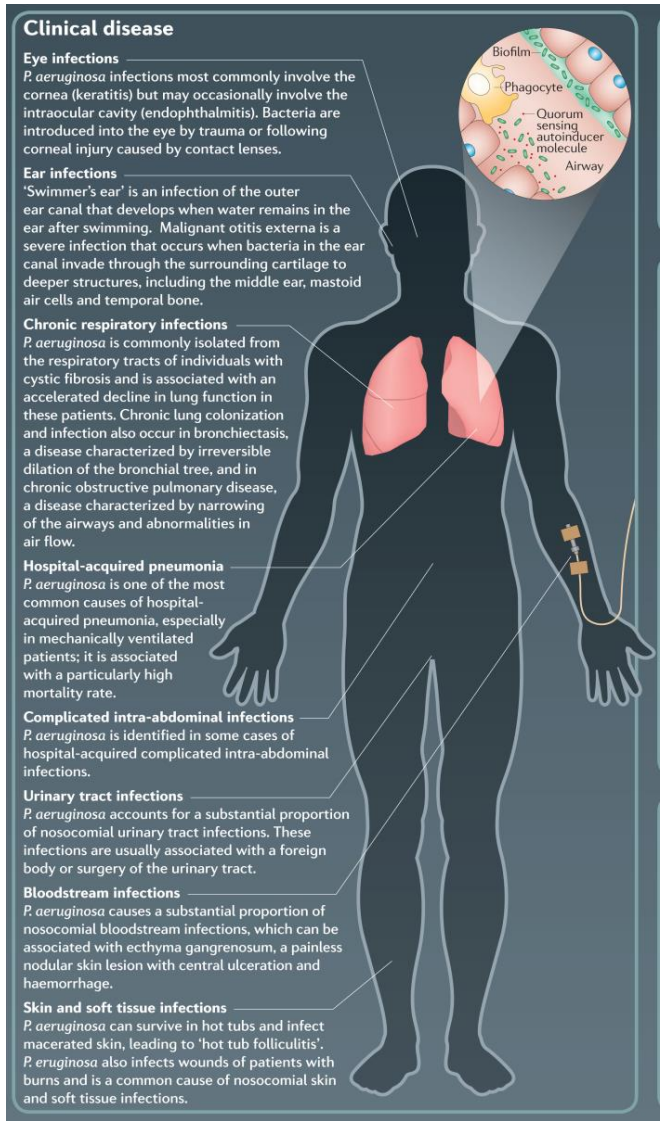


Pseudomonas aeruginosa

- **Ranked # 1 in lung infection of cystic fibrosis (CF) patients (1:2,500 newborns)**
- Ranked # 1 in intensive care unit (ICU) infections
- Ranked # 3 in non-ICU hospital-acquired (HA) infections
- Ranked # 2 in HA bacterial pneumonia
- Severity of infection
- Intrinsic antibiotic resistance
- Acquired antibiotic resistance (HGT)
- **Pan-resistance in nosocomial and CF strains**





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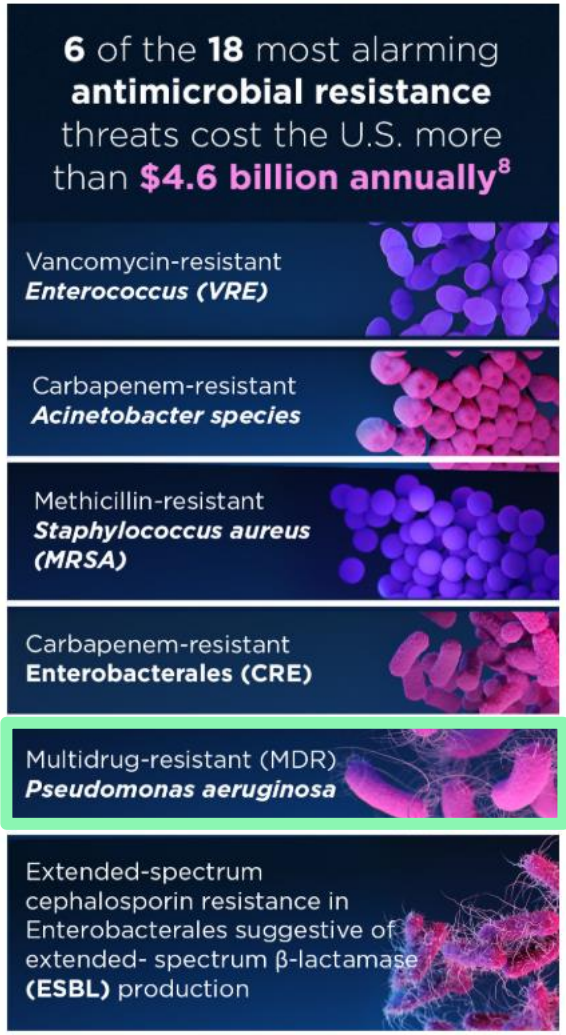
Need for new therapeutic options



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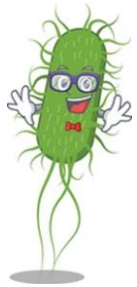
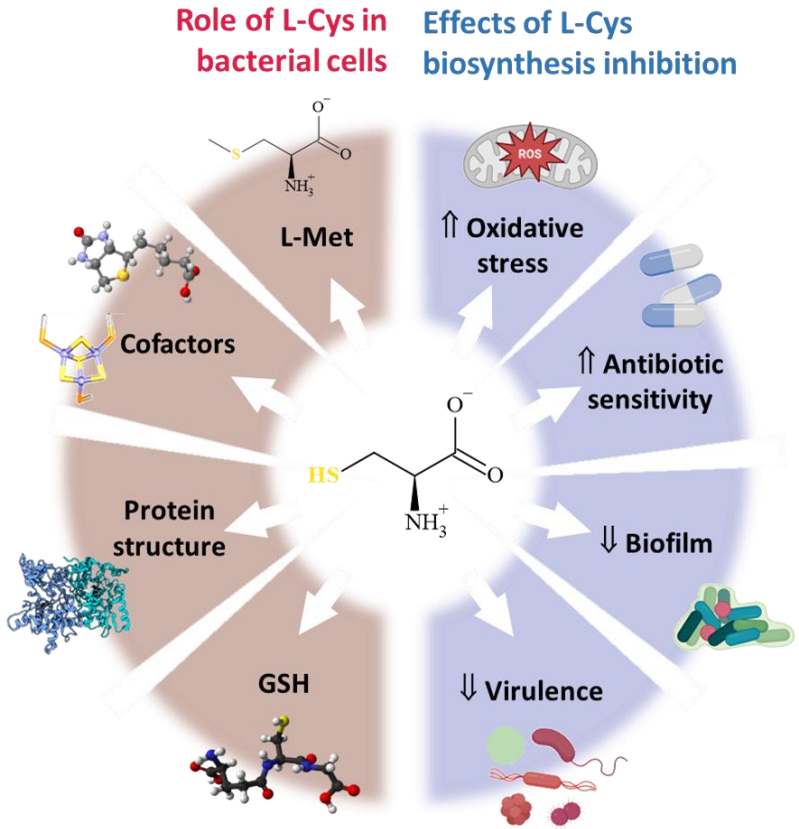
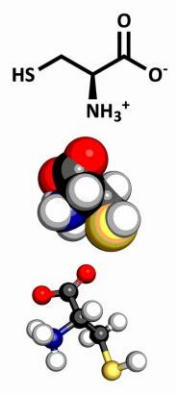
L-cysteine is fundamental for many biological processes



Pseudomonas aeruginosa



L-cysteine



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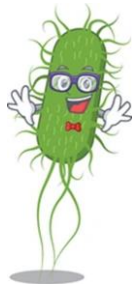
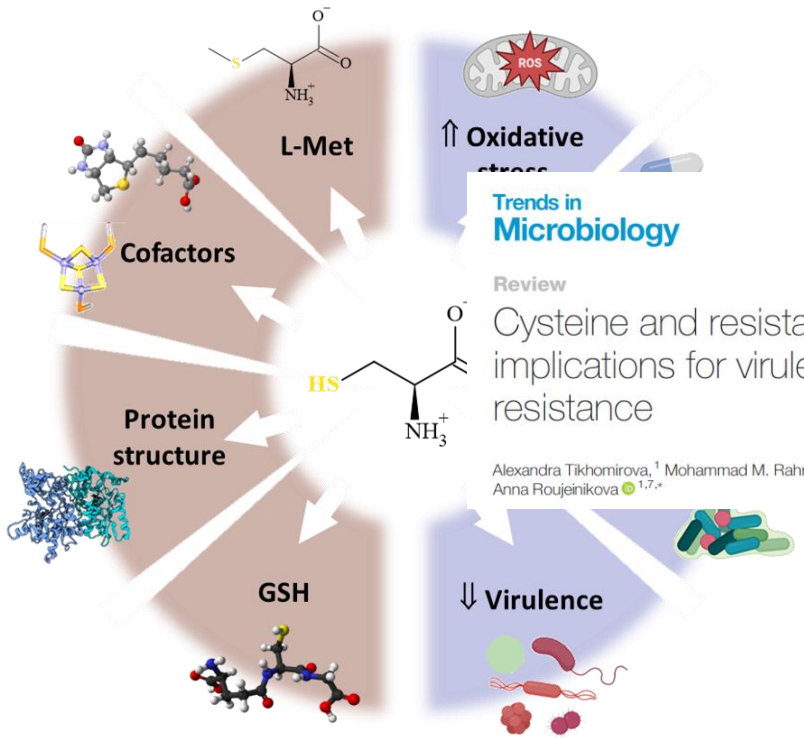


Pseudomonas aeruginosa



Trends in Microbiology

Review

Cysteine and resistance to oxidative stress: implications for virulence and antibiotic resistance

Alexandra Tikhomirova,[1] Mohammad M. Rahman,[2] Stephen P. Kidd,[3,4] Richard L. Ferrero,[1,5,6] and Anna Roujeinikova[1,7,*]

ACS chemical biology

pubs.acs.org/acschembio

First-in-Class Inhibitors of Sulfur Metabolism with Bactericidal Activity against Non-Replicating *M. tuberculosis*

Prakash B. Palde,[†] Ashima Bhaskar,[‡] Laura E. Pedró Rosa,[§] Franck Madoux,[§] Peter Chase,[§] Vinayak Gupta,[†] Timothy Spicer,[§] Louis Scampavia,[§] Amit Singh,[‡] and Kate S. Carroll*[,†]

[†]Department of Chemistry, The Scripps Research Institute, Jupiter, Florida 33458, United States
[‡]Department of Microbiology and Cell Biology (MCBL), Center for Infectious Disease Research (CIDR), Indian Institute of Science (IISc.), Bangalore 560012, India
[§]Lead Identification Division, The Scripps Research Institute, Jupiter, Florida 33458, United States

CellPress

Journal of Medicinal Chemistry

Inhibitors of the Cysteine Synthase CysM with Antibacterial Potency against Dormant *Mycobacterium tuberculosis*

Katharina Brunner,[†,#] Selma Maric,[†,#] Rudraraju Srilakshmi Reshma,[‡] Helena Almqvist,[§] Brinton Seashore-Ludlow,[§] Anna-Lena Gustavsson,[§] Ömer Poyraz,[†] Perumal Yogeeswari,[‡] Thomas Lundbäck,[§] Michaela Vallin,[§] Dharmarajan Sriram,[⊥,‡] Robert Schnell,[⊥,†] and Gunter Schneider*[,†]

[†]Division of Molecular Structural Biology, Department of Medical Biochemistry & Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden
[‡]Drug Discovery Research Laboratory, Department of Pharmacy, Birla Institute of Technology & Science-Pilani, Hyderabad Campus, Shameerpet, R.R. District, Hyderabad 500078, Andhra Pradesh India
[§]Chemical Biology Consortium Sweden, Science for Life Laboratory, Division of Translational Medicine & Chemical Biology, Department of Medical Biochemistry & Biophysics, Karolinska Institutet, S-171 65 Solna, Sweden

pharmaceuticals

MDPI

Article

Discovery of Substituted (2-Aminooxazol-4-yl)Isoxazole-3-carboxylic Acids as Inhibitors of Bacterial Serine Acetyltransferase in the Quest for Novel Potential Antibacterial Adjuvants

Joana Magalhães [1], Nina Franko [2], Samanta Raboni [2,3], Giannamaria Annunziato [1,4], Päivi Tammela [5], Agostino Bruno [1], Stefano Bettati [3,6,7], Stefano Armao [2], Costanza Spadini [8], Clotilde Silvia Cabassi [8], Andrea Mozzarelli [2,3,7], Marco Pieroni [1,4,*], Barbara Campanini [2] and Gabriele Costantino [1,4]

ACS Infectious Diseases

pubs.acs.org/journal/aidcbc

Article

Investigational Studies on a Hit Compound Cyclopropane–Carboxylic Acid Derivative Targeting *O*-Acetylserine Sulfhydrylase as a Colistin Adjuvant

Giannamaria Annunziato,*[,○] Costanza Spadini,[○] Nina Franko, Paola Storici, Nicola Demitri, Marco Pieroni, Sara Flisi, Lucrezia Rosati, Mattia Iannarelli, Marialaura Marchetti, Joana Magalhaes, Stefano Bettati, Andrea Mozzarelli, Clotilde Silvia Cabassi, Barbara Campanini, and Gabriele Costantino

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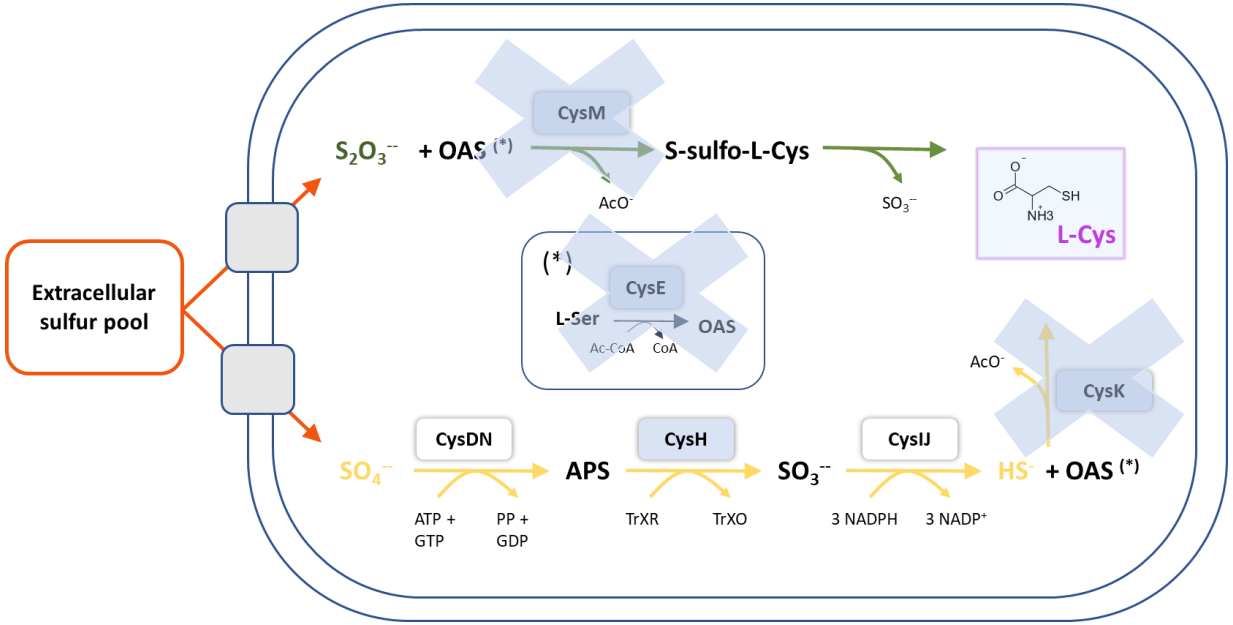
Italiadomani PIANO NAZIONALE DI RIPRESA E RESILIENZA

L-cysteine metabolism as target for antibiotic development

Bacteria, differently from humans, are able to synthesise aminoacids, including L-Cys, from inorganic salts.
The pathway has been characterized in Mycobacteria and some gram-negative bacteria…
…but it has been overlooked in ***Pseudomonas aeruginosa***



ENHANCE
dE Novo L-cysteine biosynthesis in *Pseudomonas aeruginosa*: patHway Assessment for novel aNtibiotic disCovEry
The project is funded by:

Martedì R., D'Angelo J., Sassi G., Marchetti M., Hijazi S., Percudani R., Bettati S., Campanini B., Frangipani E. *De novo* cysteine biosynthesis in *Pseudomonas aeruginosa*: characterization of the two main cysteine synthase isoforms. *Under review in iScience.*

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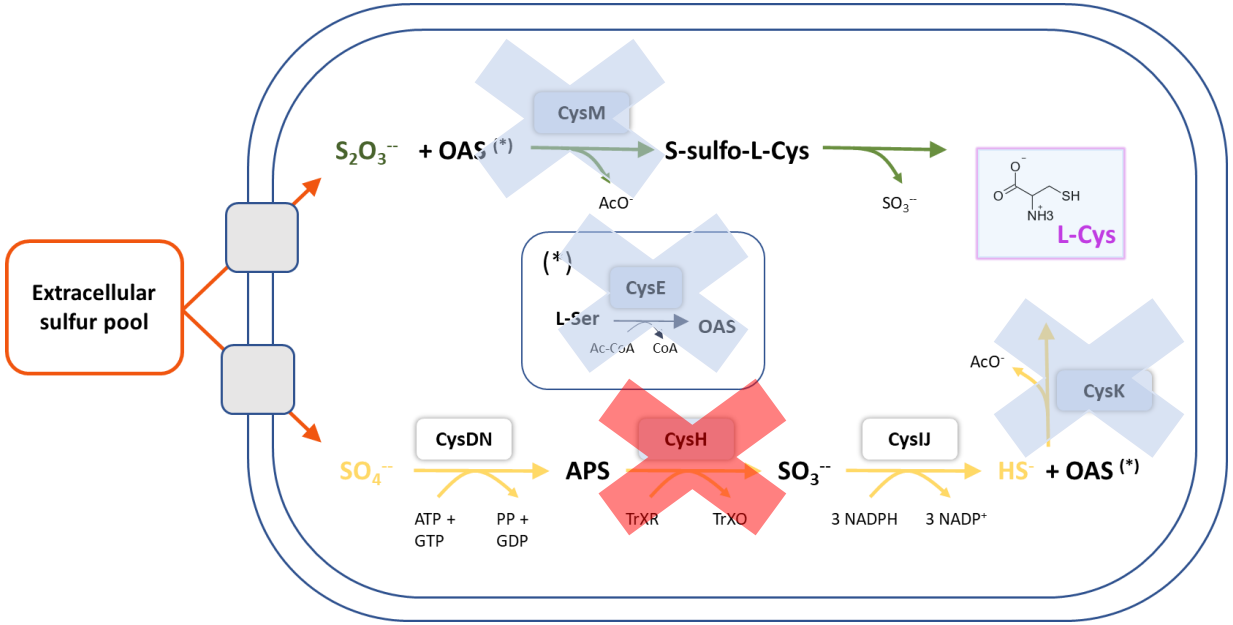

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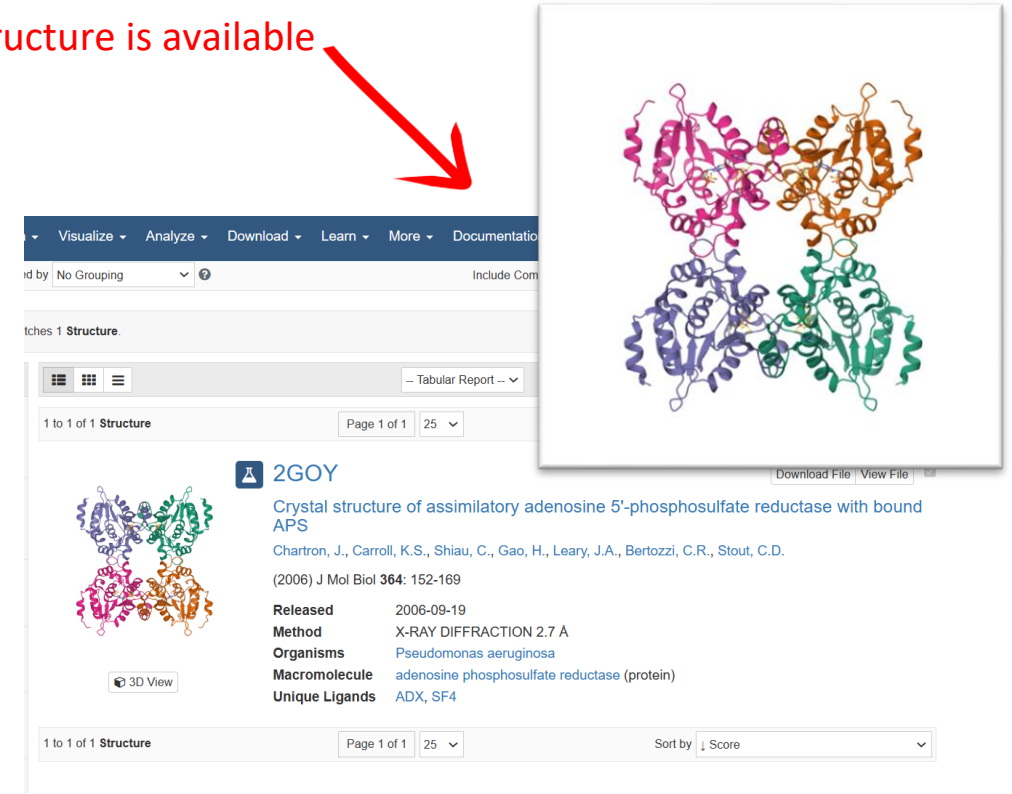
L-cysteine metabolism as target for antibiotic development

Gene	PAdb ID	Identity with *E. coli* ortholog
cysD	PA4443	78 %
cysN	PA4442	56 %
cysH	PA1756	27 %
cysI	PA1838	22 %
cysJ	PA4513	32 %
cysE	PA3816	40 %
cysK	PA2709	71 %
cysM	PA0932	69 %

CysH crystal structure is available



How could we assay inhibitor activity

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CysH virtual screening

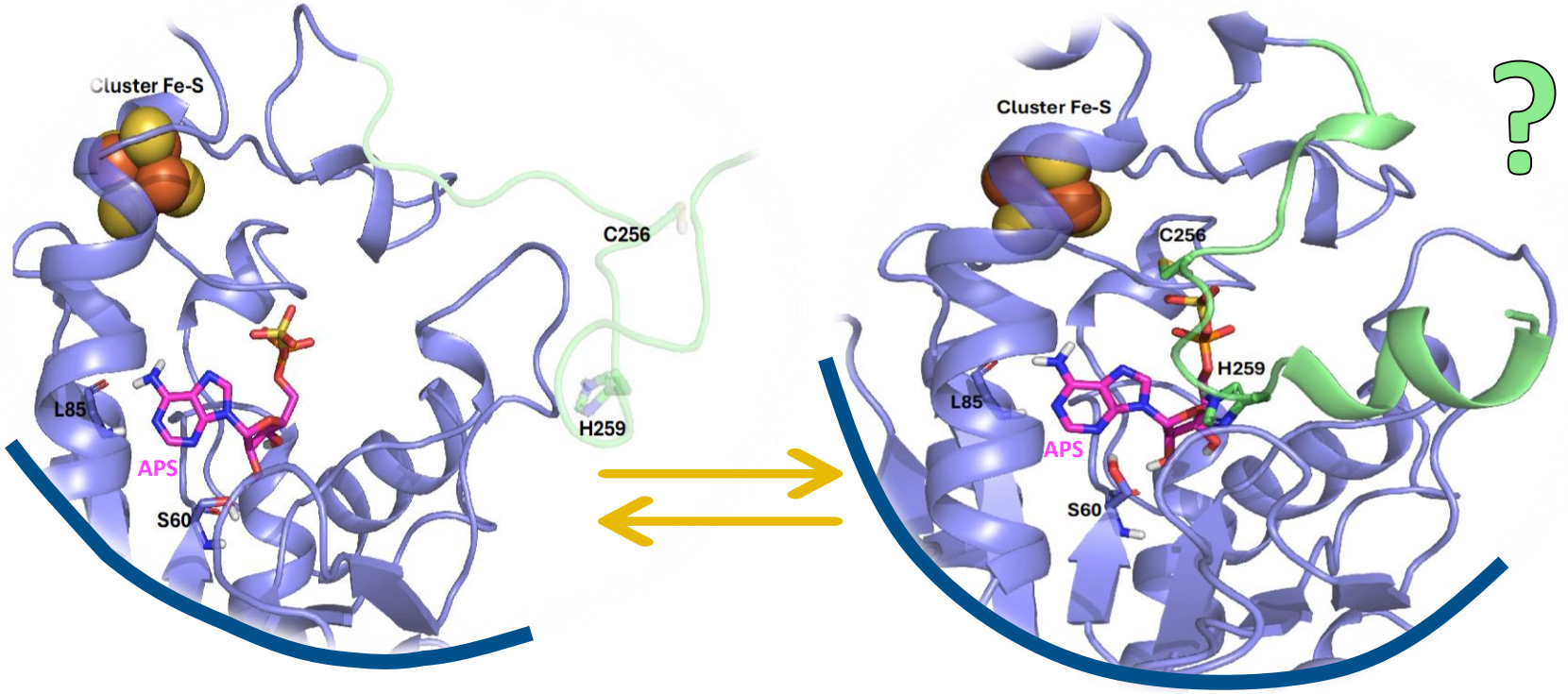
ACTIVITY

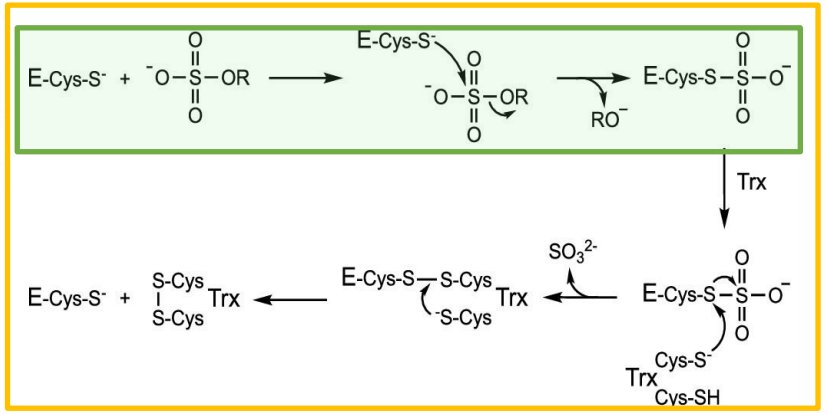
Enzyme in the open/inactive conformation

- ❑ Lack the *C*-terminal region in X-ray structures
- ❑ Missing catalytic C256

Enzyme in the closed/active conformation

- ❑ Prediction of the *C*-terminal region through AlphaFold2.





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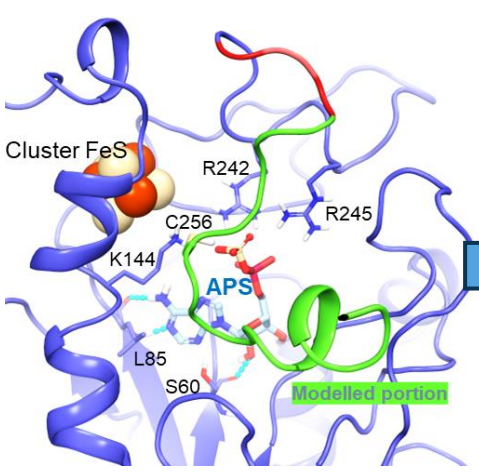
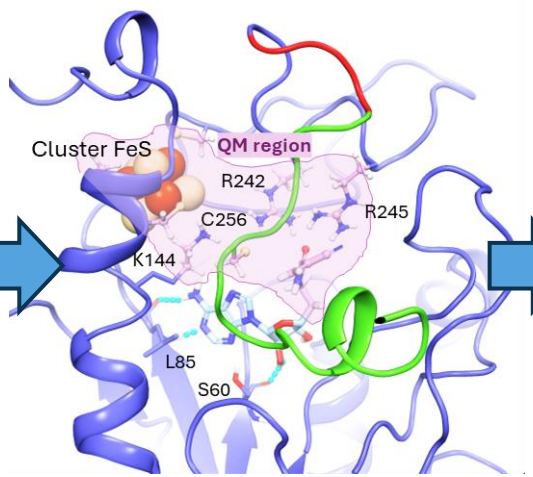
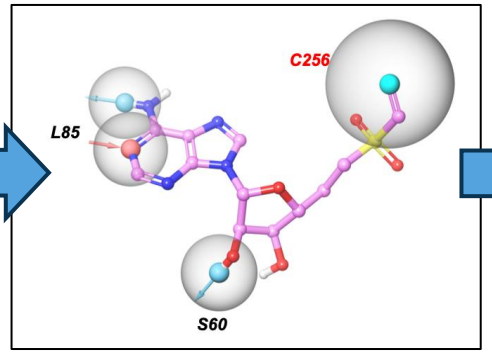
Computational workflow retrieving APSR **covalent** inhibitors



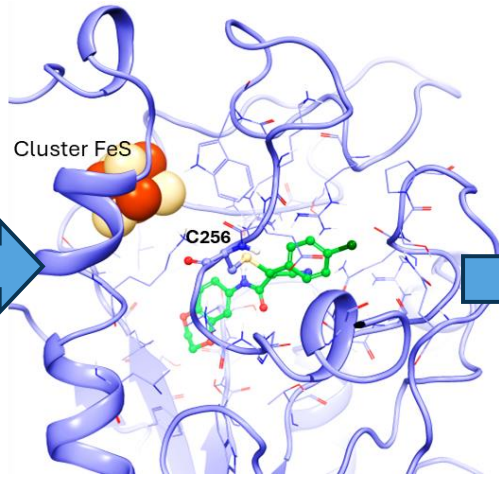
Modelling of the C-terminal loop and position of the catalytic cysteine



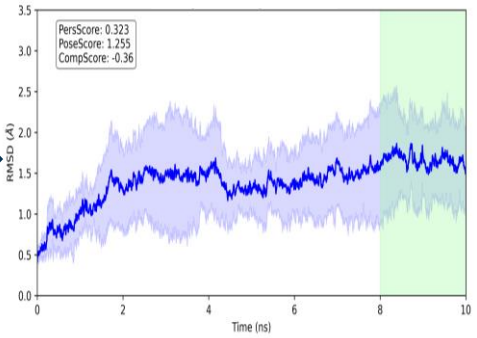
QM/MM minimizations of the Michaelis complexes retrieving optimized geometries and charges



Building of the pharmacophore model to position the reactive warhead and reproduce substrate interactions

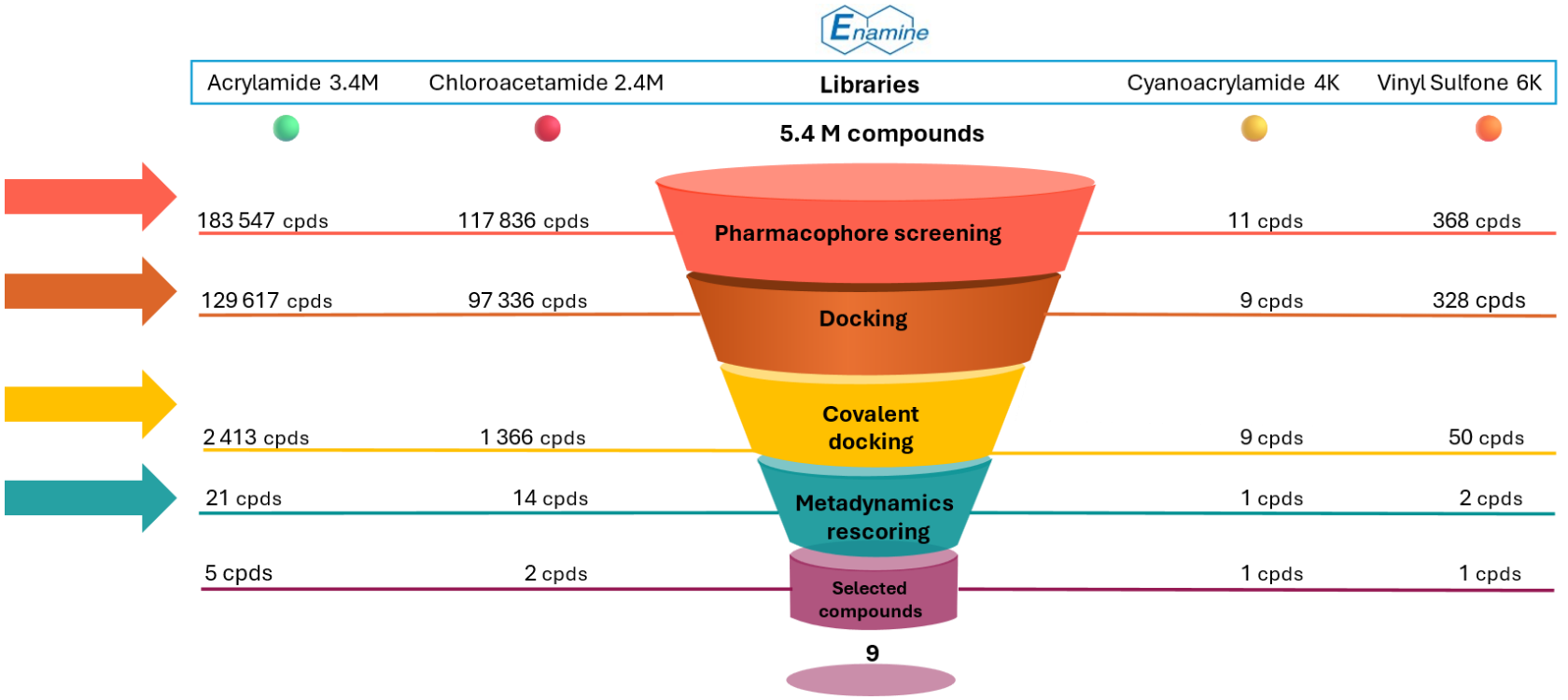


Covalent docking calculations to prioritize commercially available compounds



Well-tempered metadynamics simulations to estimate pose stability and ligand interactions

Results of virtual screening protocol



Strategies

- ❑ Retrieval of warheads with varied reactivity
- ❑ Acceleration of covalent docking via starting reactive docking poses
- ❑ Prioritization of compounds with Binding-Pose Metadynamics, prioritizing stable binding modes (RMSD<2Å)

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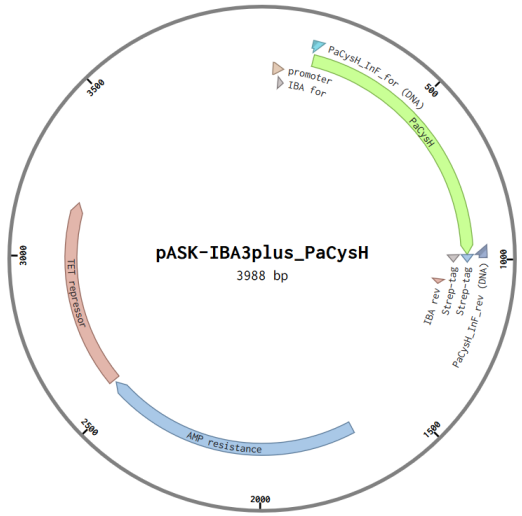
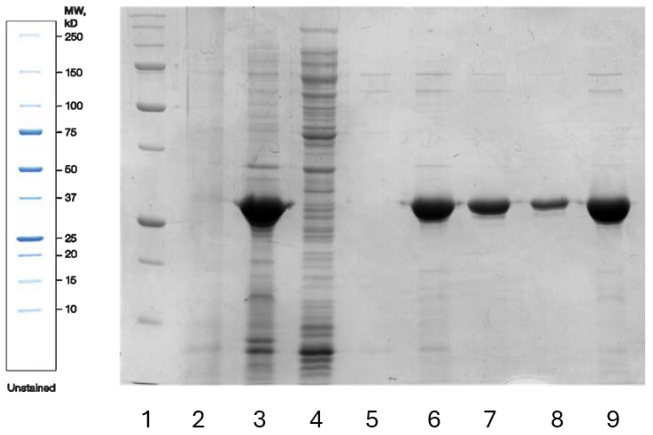
$CysH_{Pa}$ activity (and inhibition) can be investigated through different *in vitro* assays

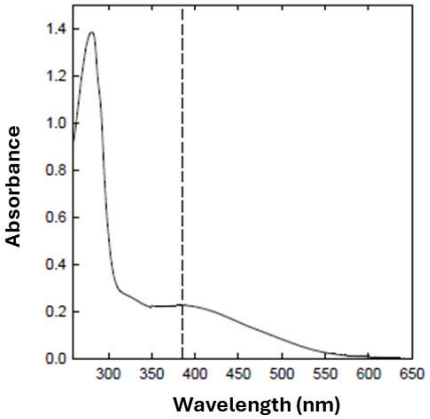
Protein recombinant production in *E. coli* (C-terminal Strep-tag). Purification under **aerobic conditions**, good yields (30 mg/L)

O05927 · CYSH_PSEAE
https://www.uniprot.org/uniprotkb/O05927/entry
MLPFATIPATERNSAAQHQDPSPMSQPFDLPALASSLADKSPQDILKA
AFEHFGDELWISFSGAEDVVLVDMAWKLNRNVKVFSLDTGRLHPETYR
FIDQVREHYGIAIDVLSPDPRLLEPLVKEKGLFSFYRDGHGECCGIRK
IEPLKRKLAGVRAWATGQRRDQSPGTRSQVAVLEIDGAFSTPEKPLYK
FNPLSSMTSEEVWGYIRMLELPYNSLHERGYISIGCEPCTRPVLPNQH
EREGRWWWEEATHKECGLHAGNLISKAENLYFQ↓SAWSHPQFEK

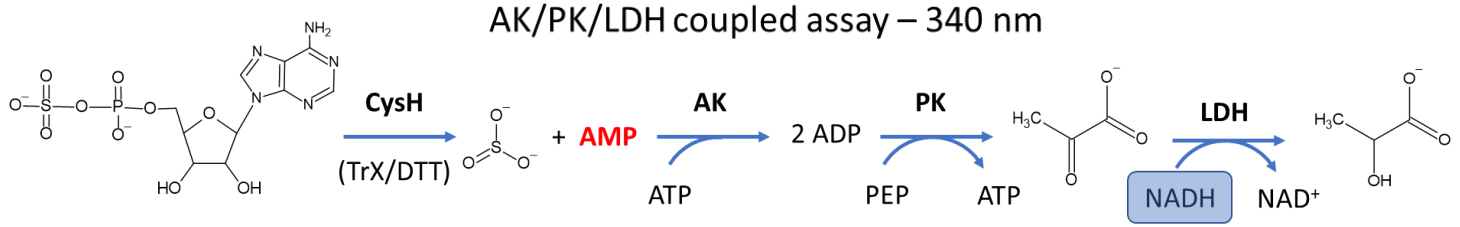




1- marker
2- insoluble fraction
3- soluble fraction
4- flow through
5- wash
6- elution 1
7- elution 2
8- elution 3
9- final preparation

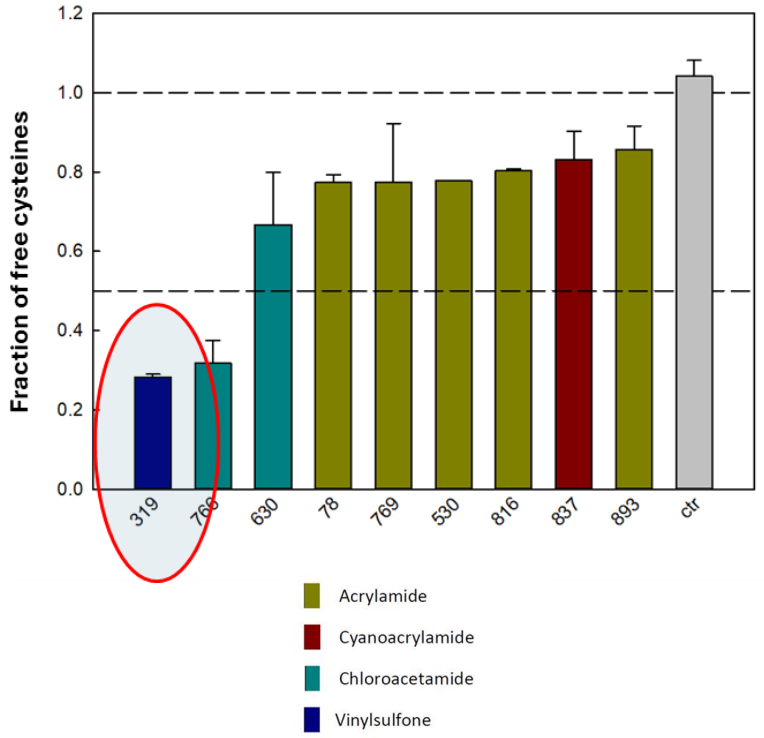


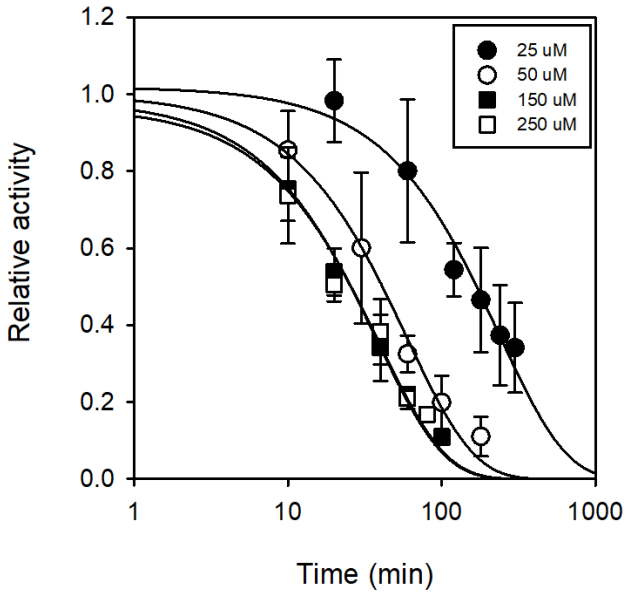
AMP detection:
AK/PK/LDH coupled assay – 340 nm



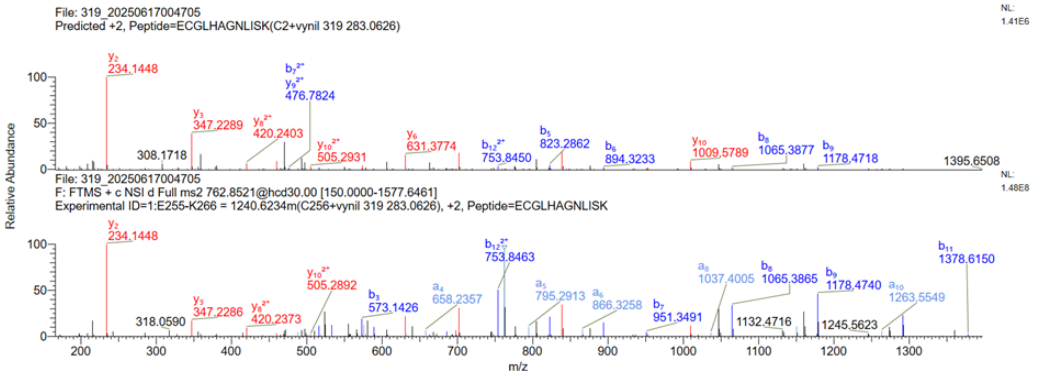
λ_{max} = 340 nm

CysH is catalytically active and forms a covalent adduct with 319 that involves the catalytic cysteine





MS studies confirm selective vinylation of C256 through fragmentation patterns of peptide E[C-vinyl]GLHAGNLISK



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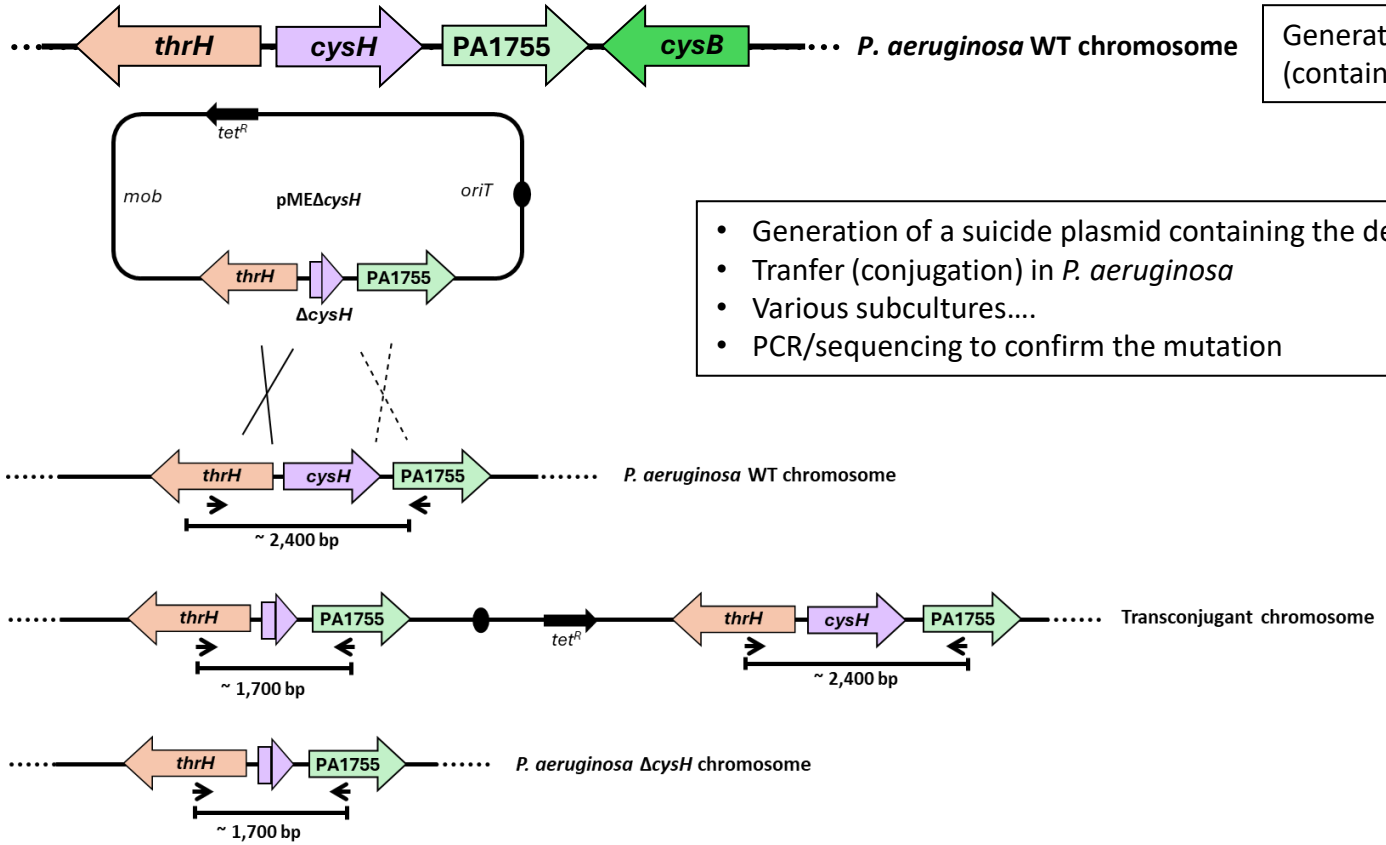
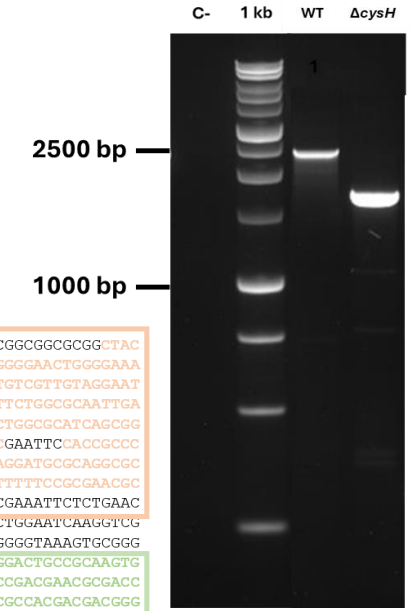
What we have done about **CysH** in *Pseudomonas aeruginosa*…

We have successfully generated a ***cysH* in-frame deletion mutant** and characterized its phenotype



Generation of the deletion fragment
(containing the upstream and the downstream regions of *cysH* gene)

- Generation of a suicide plasmid containing the deletion of interest
- Tranfer (conjugation) in *P. aeruginosa*
- Various subcultures….
- PCR/sequencing to confirm the mutation



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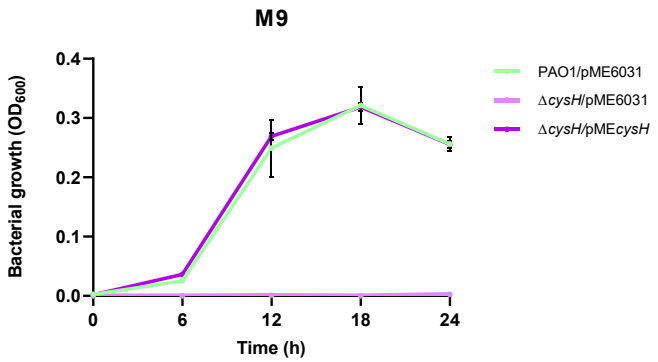
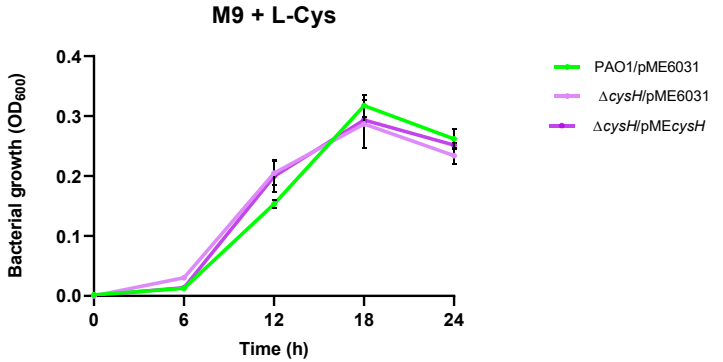
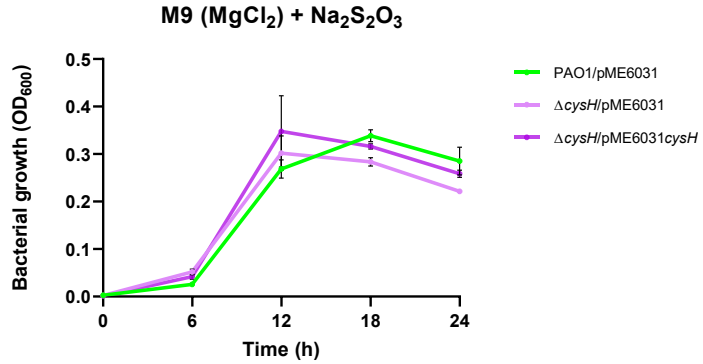
What we have done about **CysH** in *Pseudomonas aeruginosa*…

- We successfully set-up the assay conditions
  Growth in the M9 medium ± L-cysteine 40 µg/ml (optimal L-cysteine concentration able to restore the growth of the *cysH* deletion mutant) can be considered as suitable test conditions for putative CysH-inhibitors
- We have genetically complemented the *cysH* deletion mutant

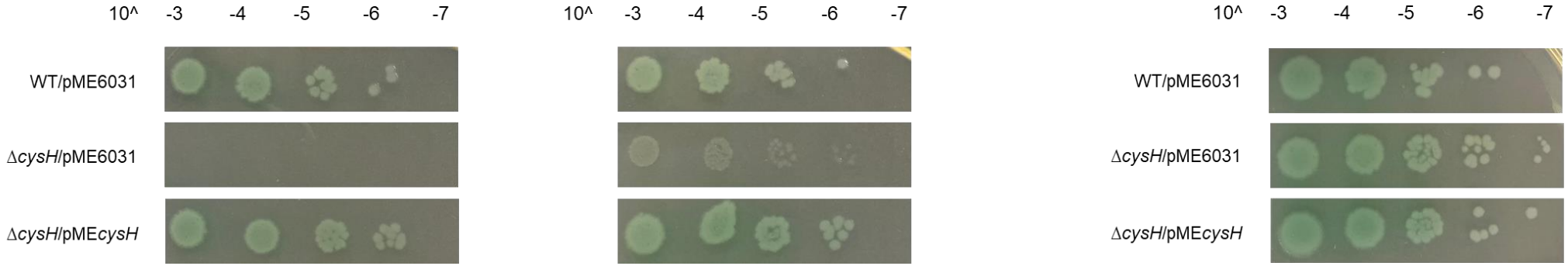
Liquid medium







Solid medium



Prof. Emanuela Frangipani – Rebecca Martedì - Dr. Sarah Hijazi

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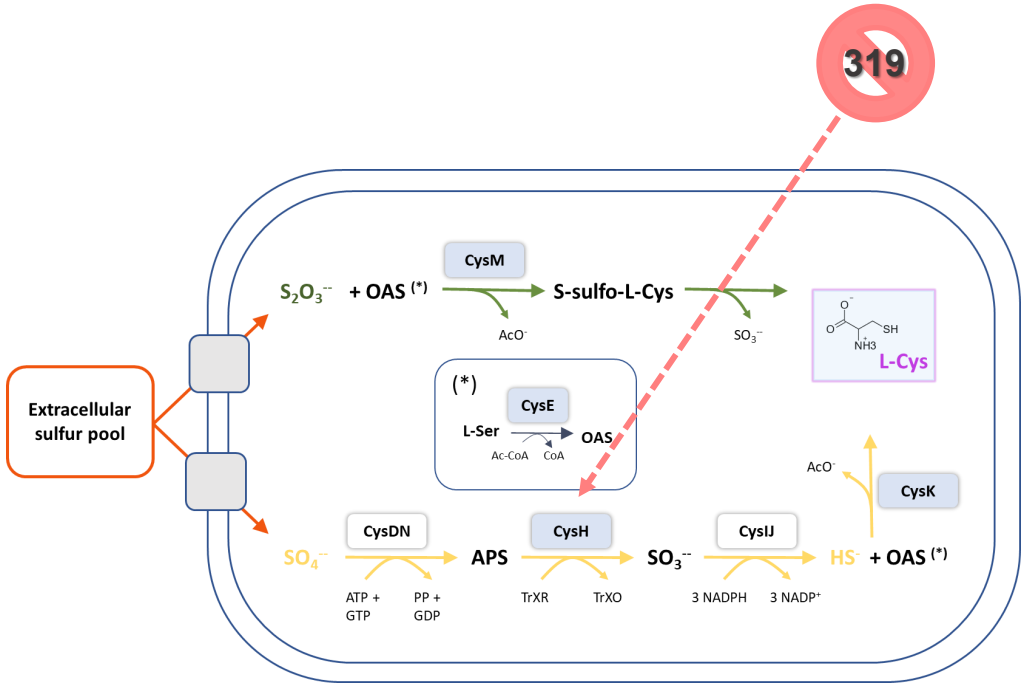
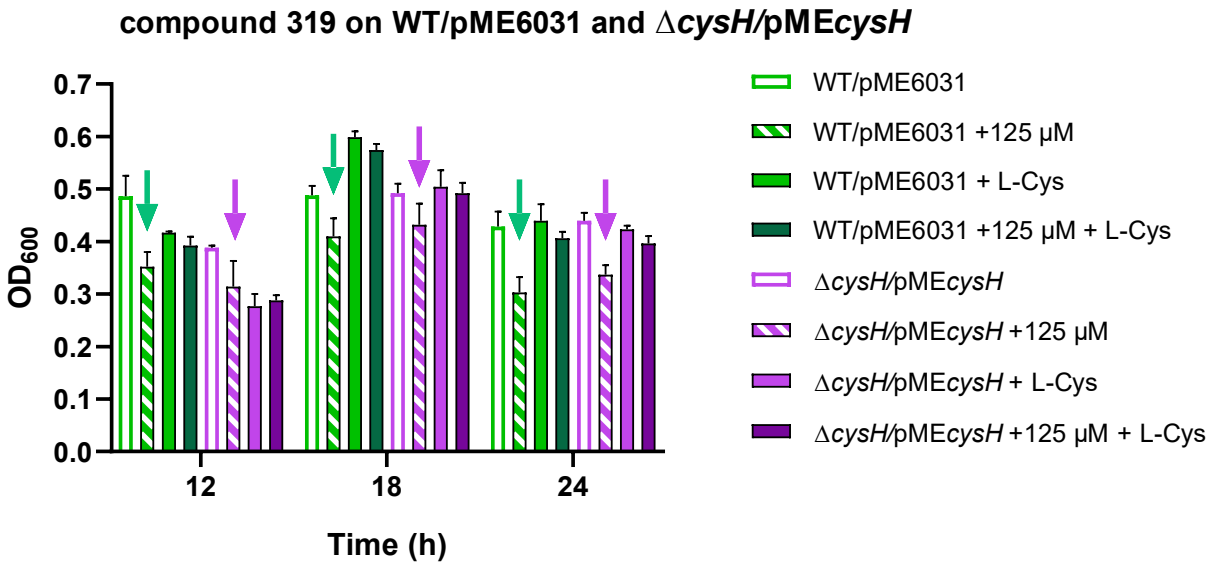
CysH inhibitor assay to impair L-cysteine synthesis, hence *P. aeruginosa* growth





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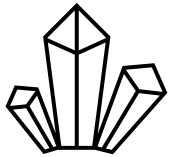
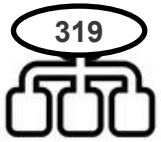
Conclusions and future perspectives

- We have successfully identified a lead compound targeting *P. aeruginosa* CysH with good activity *in vitro* and in bacterial cells, in line with the primary aim of WP1 (*Development of a third party-accessible, enabling platform for efficient preclinical drug discovery*)



- We plan to solve CysH crystal structure in complex with 319 to gain insights on the functional and catalytic properties of the C-terminal region (in collaboration with Yvain Nicolet, IBS, Grenoble) and guide further med chem efforts

- Further validate the microbiological data on 319 by increasing the number of experimental replicates



- Assay 319 derivatives that possess the same warhead to investigate a putative increase in efficacy

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1506 UNIVERSITÀ DEGLI STUDI DI URBINO CARLO BO

UNIVERSITÀ DI PARMA

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Barbara Campanini
Stefano Bettati
Marialaura Marchetti
Francesco Guggino

ENHANCE
dE Novo L-cysteine biosynthesis in *Pseudomonas aeruginosa*: patHway Assessment for novel aNtibiotic disCovEry

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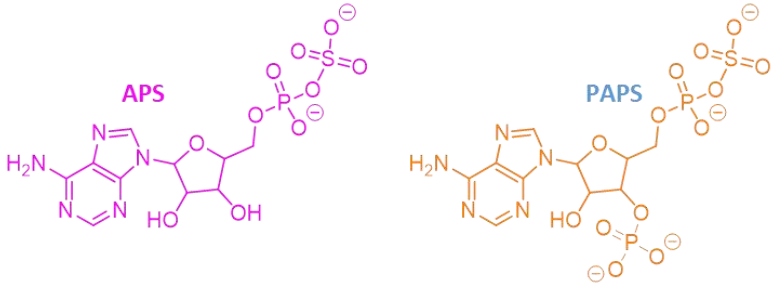
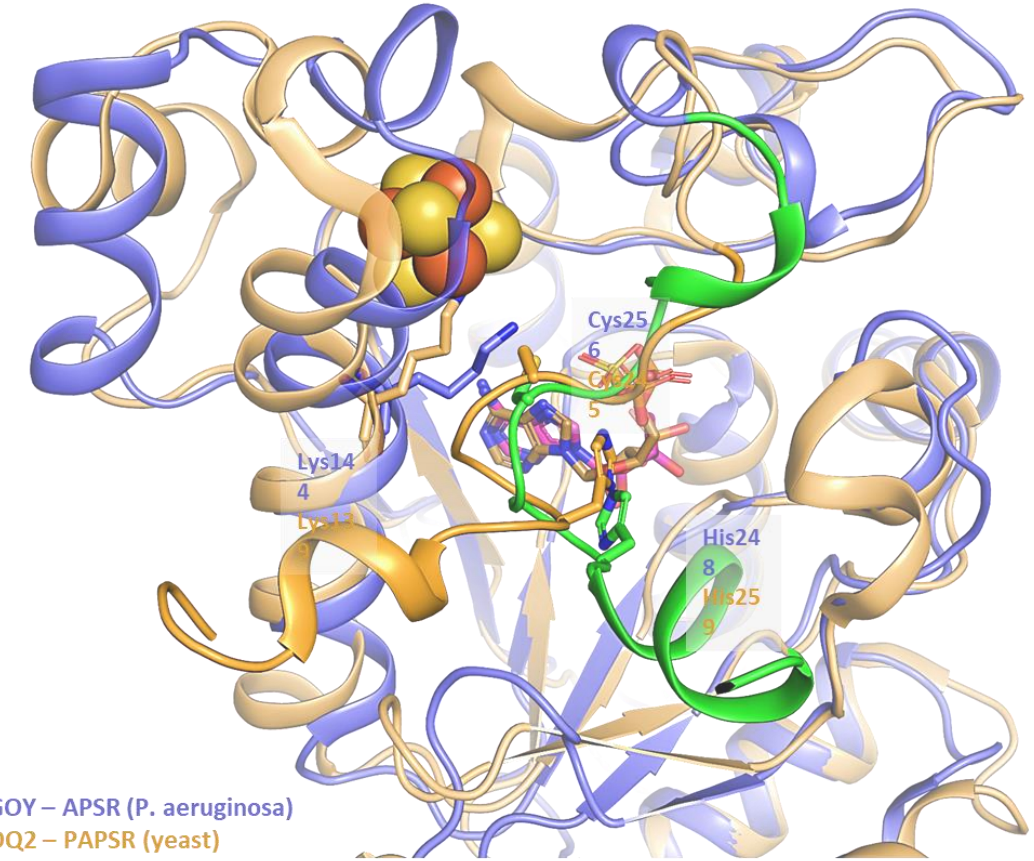
MODELING OF THE CATALYTIC PORTION OF APSR

- The **folding of the C-terminal tail** was predicted from its amino acid sequence using AlphaFold2.
- The **catalytic cysteine** is close to the sulfur atom of APS and to basic residues, such as K144 and arginine residues, that may increase cysteine nucleophilic proprieties.
- Comparison with **PAPS reductase**:
  - Conserved residues (ECG(I/L)H motif) are similarly positioned
  - Given the absence of the cluster Fe-S in the PAPS structure, its role may not be relevant for APSR mechanism.





2GOY – APSR (P. aeruginosa)
2OQ2 – PAPSR (yeast)