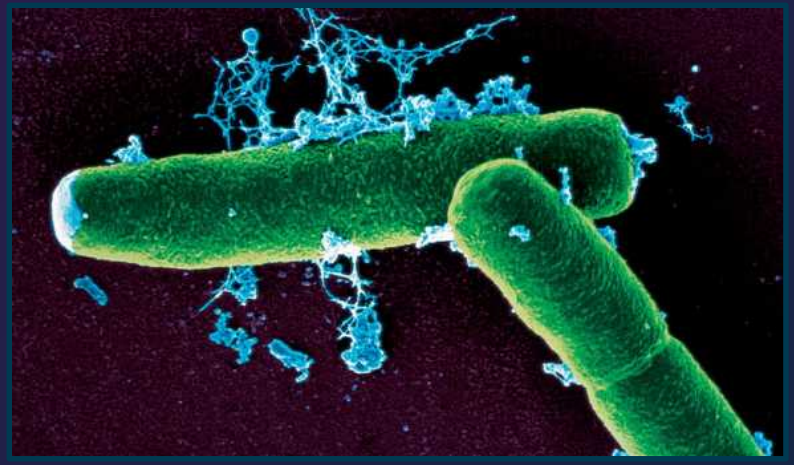
Old Enzyme With New Function.

(Organophosphate Hydrolase Exists as Part of Ton Complex and Plays a Role in Iron Transport)



by

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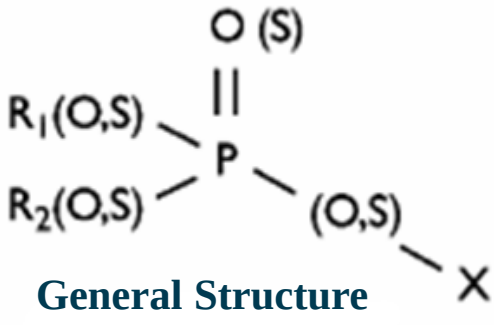
Hyderabad, India

Organophosphates (OPs): History and Structural Diversity

- **Organophosphates are esters or thiols derived from phosphoric, phosphoramidic or phosphonic acid.**



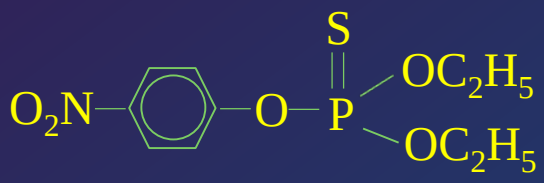
General Structure

P- (phosphinates)
O- (phosphates)
S- (phosphothioates)
R_1 and **R_2** aryl or alkyl group
X- aliphatic, aromatic, heterocyclic or halide

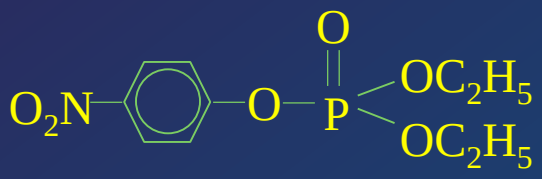
- **First synthesized in early 40's by German scientist Gerhard Schrader.**
- **As of 2000's there were 195 different OP's available in different formulations.**
- **These potent acetylcholine esterase (AchE) inhibitors were introduced into environment as replacements to more persistent Organochloride insecticides like DDT, BHC, HCH etc.**

The Most Prominent OP Compounds

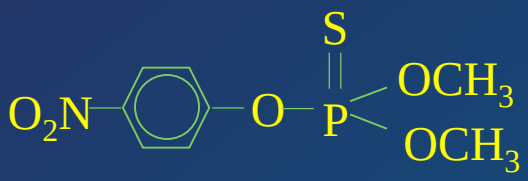
Pesticides



Parathion

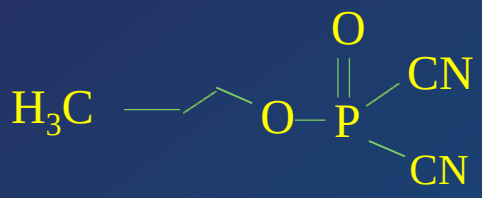


Paraoxon

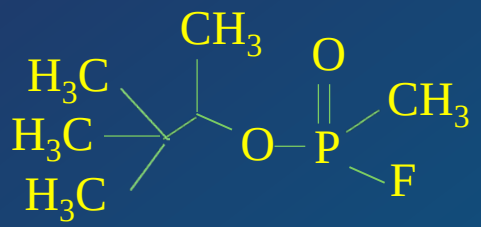


Methyl parathion

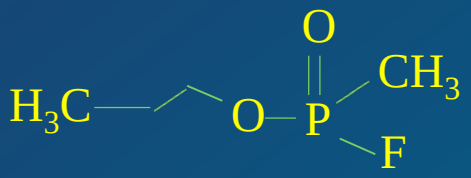
Nerve Agents



Tabun

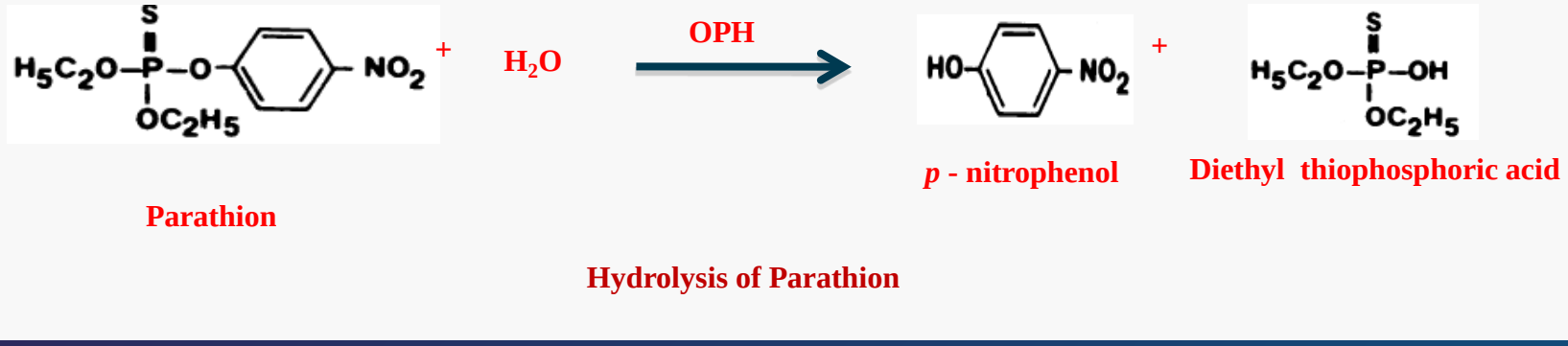


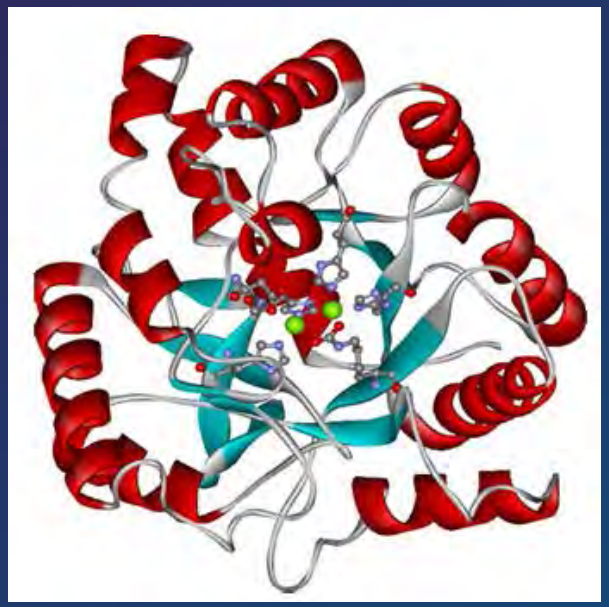
Sarin



Soman

Organophosphate Hydrolase (OPH)



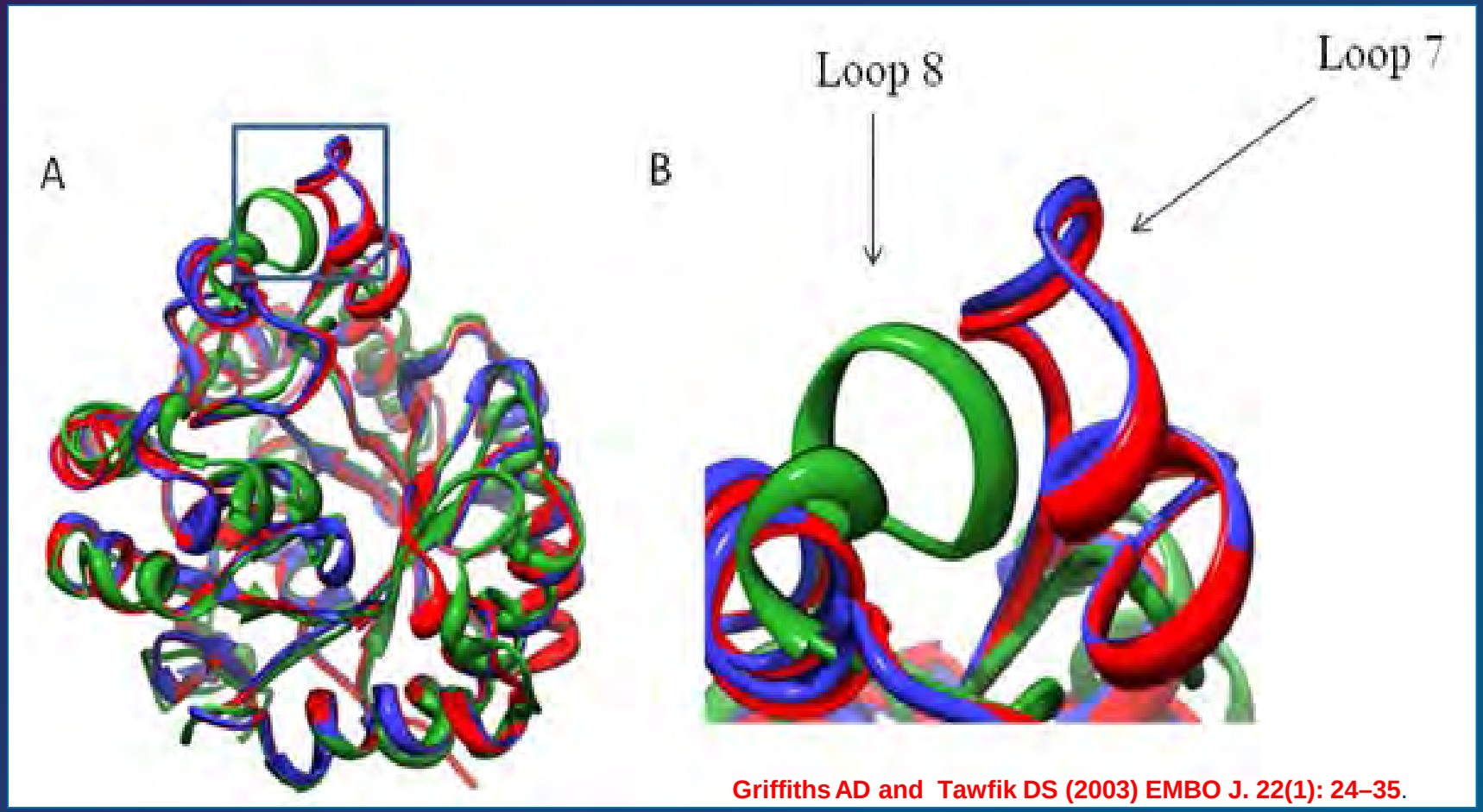


❖THE OPH HYDROLYSIS CERTAIN OPS AT A RATE THEIR
DIFFUSION LIMIT (NERVE AGENT SARIN)

❖OPH IS A DIMERIC METALLO PROTEIN HAVING TWO 36KDA MONOMERS .

Benning et al, *Biochemistry*, 1994

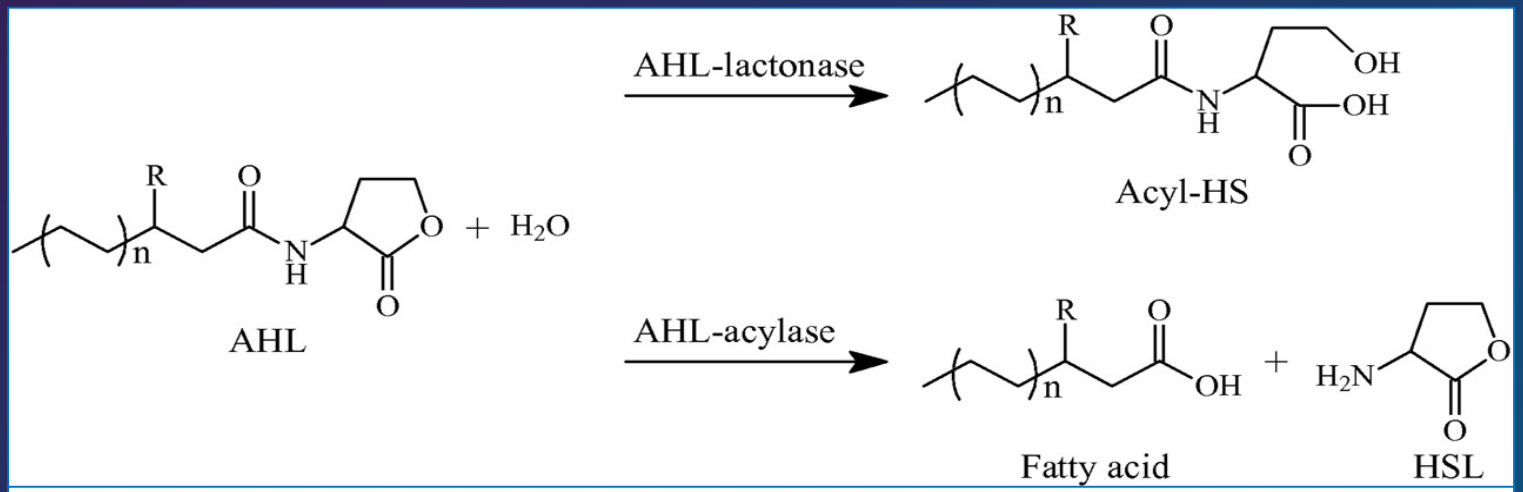
STRUCTURAL BASIS FOR NATURAL LACTONASE AND PROMISCUOUS PHOSPHOTRIESTERASE ACTIVITY

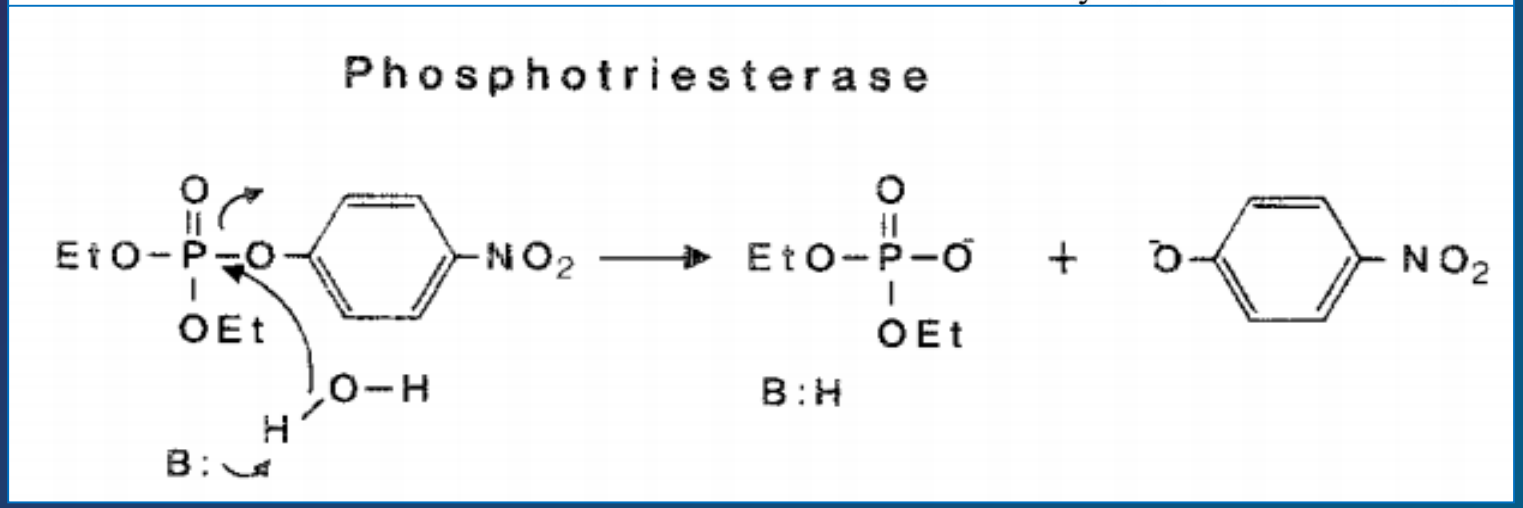


Griffiths AD and Tawfik DS (2003) EMBO J. 22(1): 24–35.

SUPERIMPOSITION OF PTES OF *B. DIMINUTA* (RED) AND *A. RADIOBACTER* (BLUE) WITH THE PHOSPHOTRIESTERASE LIKE LACTONASES (PLLS) OF *S. SOLFATARICUS* (SSOPOX), ABSENCE OF 15 RESIDUE LONG LOOP 8 IN SSOPOX IS SHOWN WITH ARROW MARK.

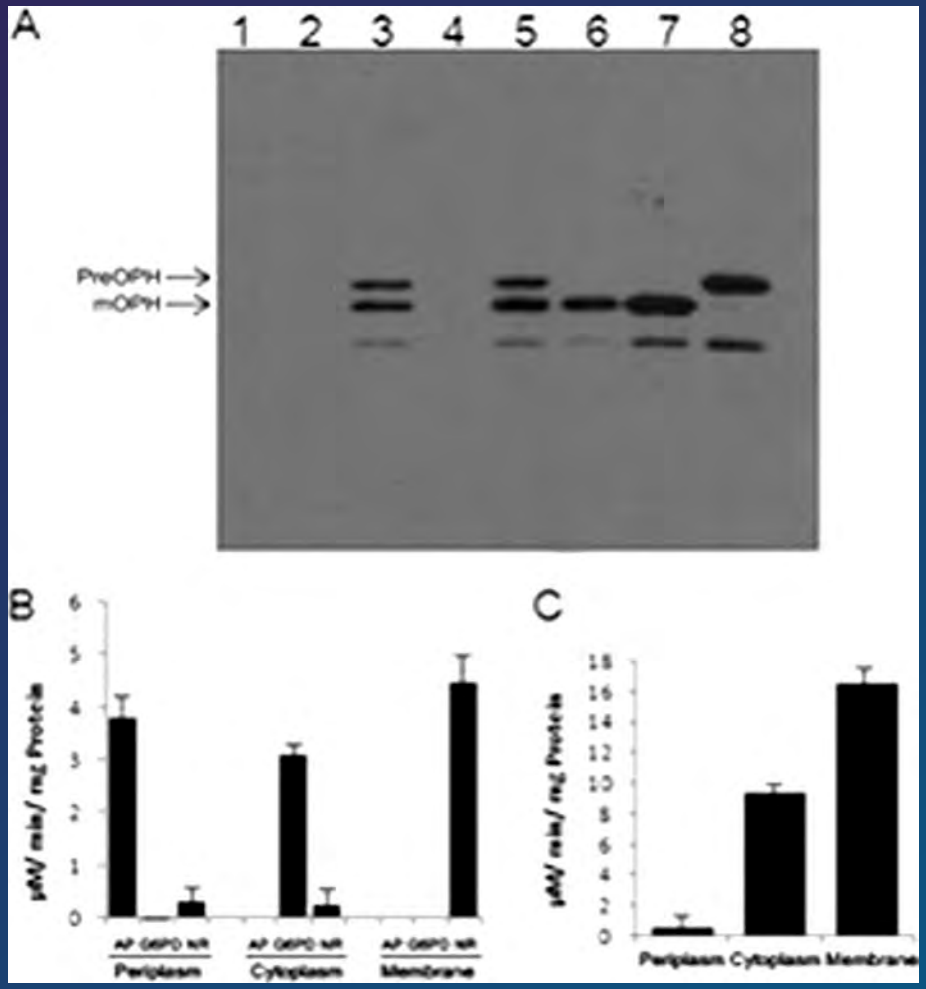
ENZYMATIC REACTIONS CATALYZED BY LACTONASES AND PHOSPHOTRIESTERASE





Sub-cellular Localization of OPH.

Question:
Where is it Located in the cell?



(A) Western blot (using anti-His antibody) to detect OPH-6His in SDS-PAGE of total cellular proteins of *B. diminuta* (opd::tet) without (lane 2) and with (lane 3) expression plasmid pSM5.

Purushotham Gorla et al. J. Bacteriol. 2009;191:6292-6299

Journal of Bacteriology

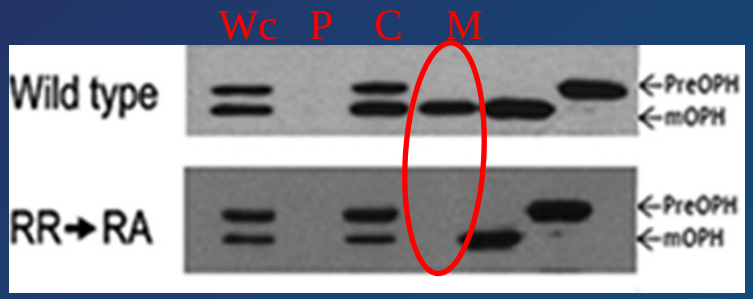
Tat motif is essential for membrane targeting of Organophosphate Hydrolase

Subcellular Fractionation

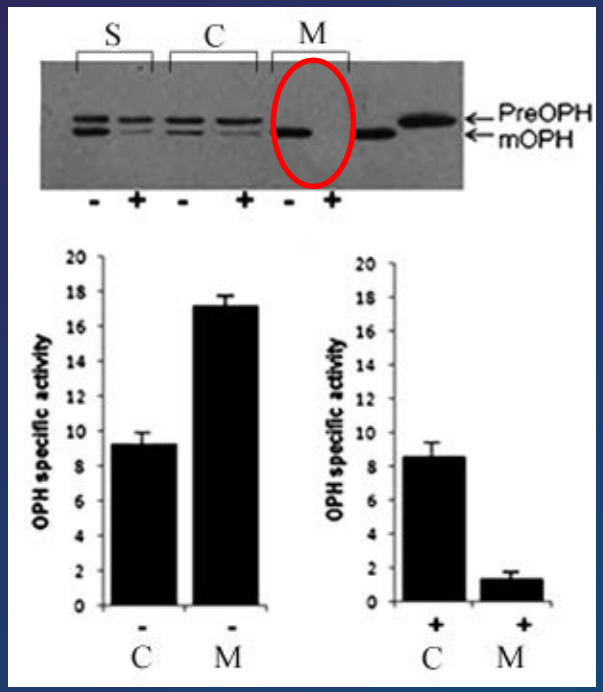
P C M

OPH is Tat substrate

MQT**RR**V**VLK**SAAAAGTLLGGLAG



Proteinase K treatment



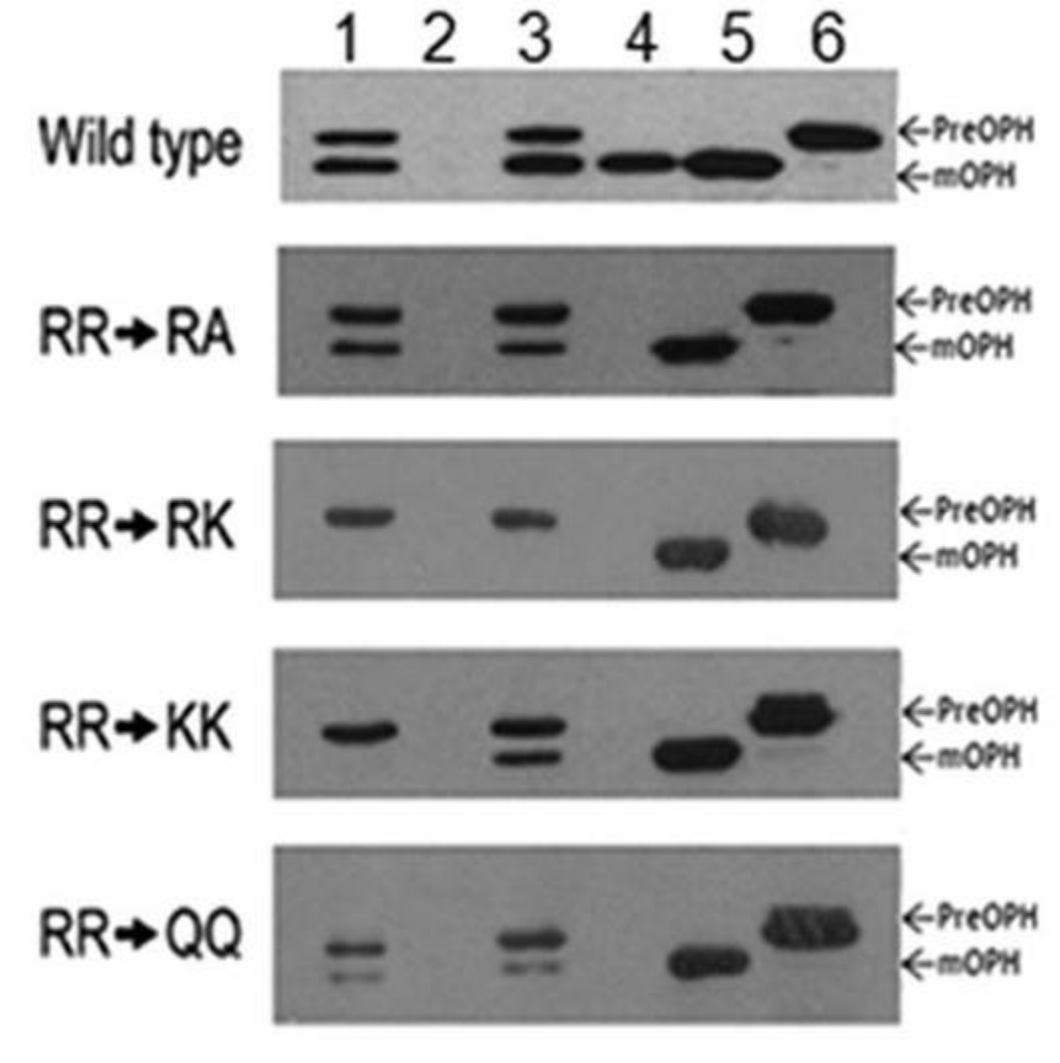
WC= Whole cells; S= Spheroplasts; P = Periplasm; C = Cytoplasm; M = Membrane.

- OPH is located in the membrane fraction.
- The Invariant arginines in the signal peptide are essential for membrane targetting.
- Proteinase K treatment of spheroplasts causes disappearance of signal and OPH activity in membrane.

Question : Is Tat motif essential for membrane targetin

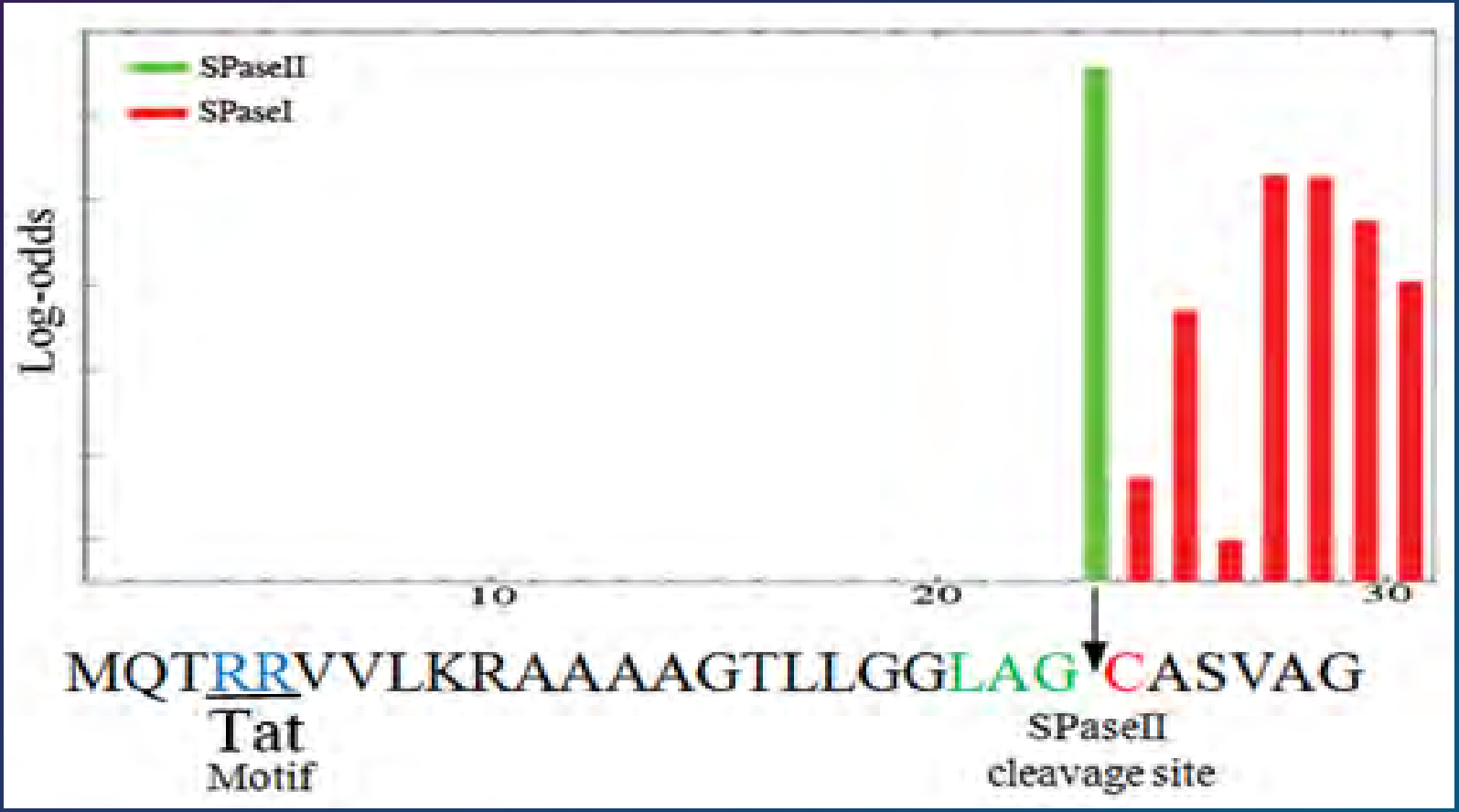
Gorla et al., 2009, *J Bacteriol* **191: 20 6292-6299**

The invariant Arginines Found in Tat Motif are Essential for Membrane Targeting of OPH.



Purushotham Gorla et al. J. Bacteriol. 2009;191:6292-6299

The OPH has a 23 Amino Acids Long Signal Peptide Which contains a Tat motif and a Lipo-Box.



- Like lipoproteins OPH has a unique signal peptide contains invariant cysteine as the +1 residue.

- The OPH has consensus Tat motif (TRRVVL) generally found in membrane proteins translocated in preforlded conformation.

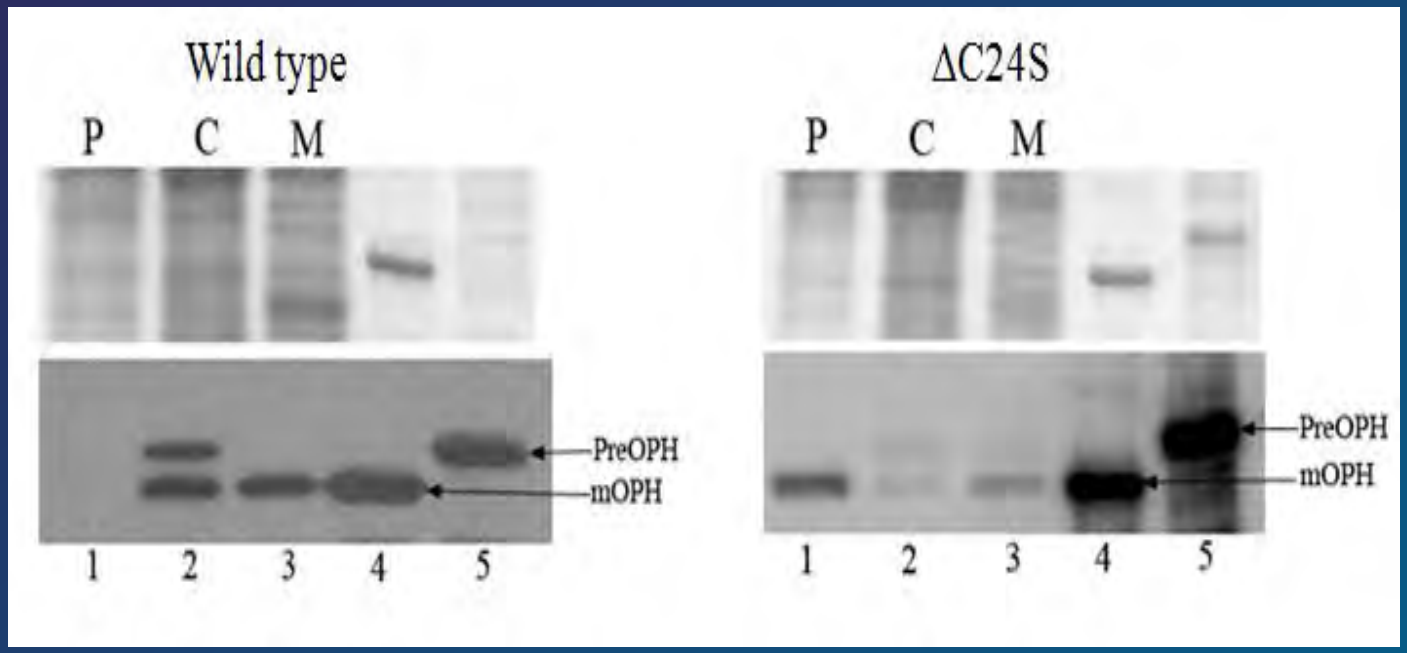
Question: Is OPH a lipoprotein?

The Invariant Cysteine Present in Lipo-Box is Essential for Membrane Anchoring

Question: How is it anchored to the membrane ?

OPH Signal Sequence

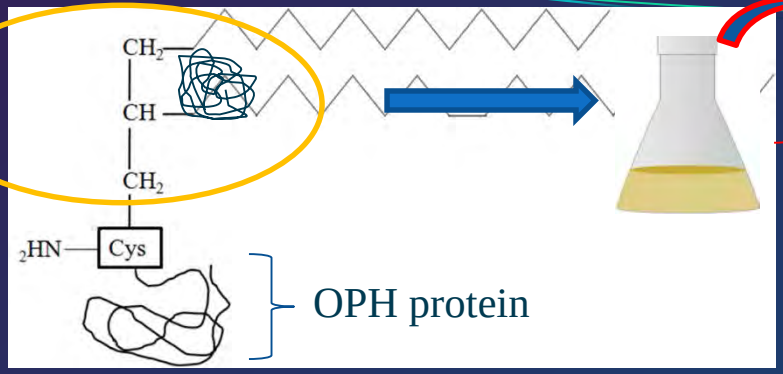
MQT**RR**VVLKSAAAAGTLLGGLAG**C**ASVAG



The Invariant Cysteine residue is important for membrane anchoring !

Parthsarathy et al, (2016) J. Biological. Chem. 291: 7774–85.

The OPH is anchored to the Inner Membrane via diacyl glycerol linked to the invariant cystein



B. diminuta Wild type

Lipid linkage

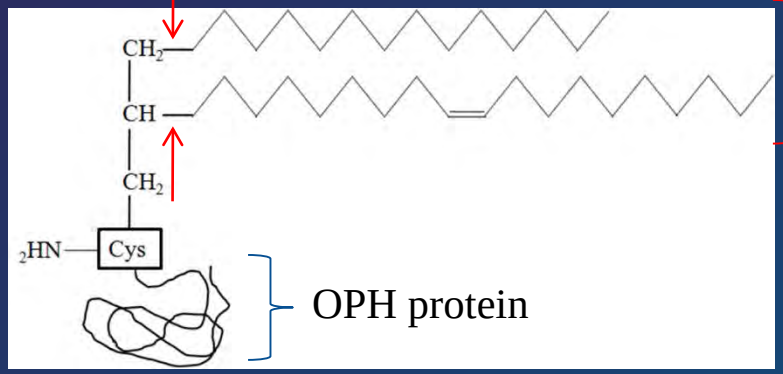
Harvest after 12h and fractionate

Solubilize the membrane with Triton X-100

Immunopurify OPH (anti-OPH column)

MeoH-HCl hydrolysis and FAME prep

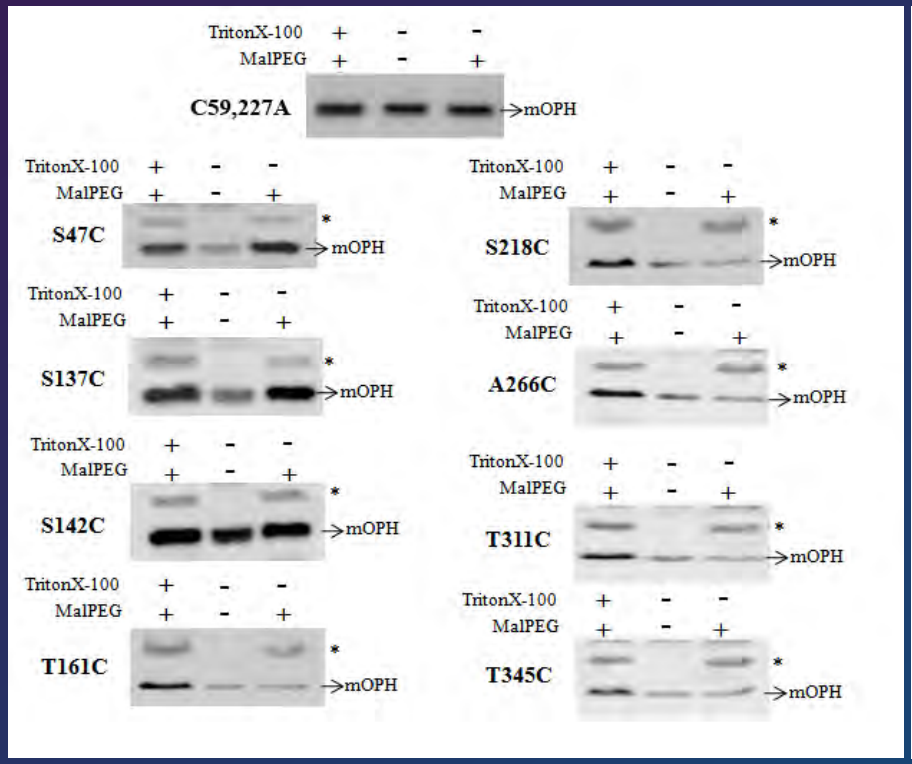
GC/MS to identify the lipids

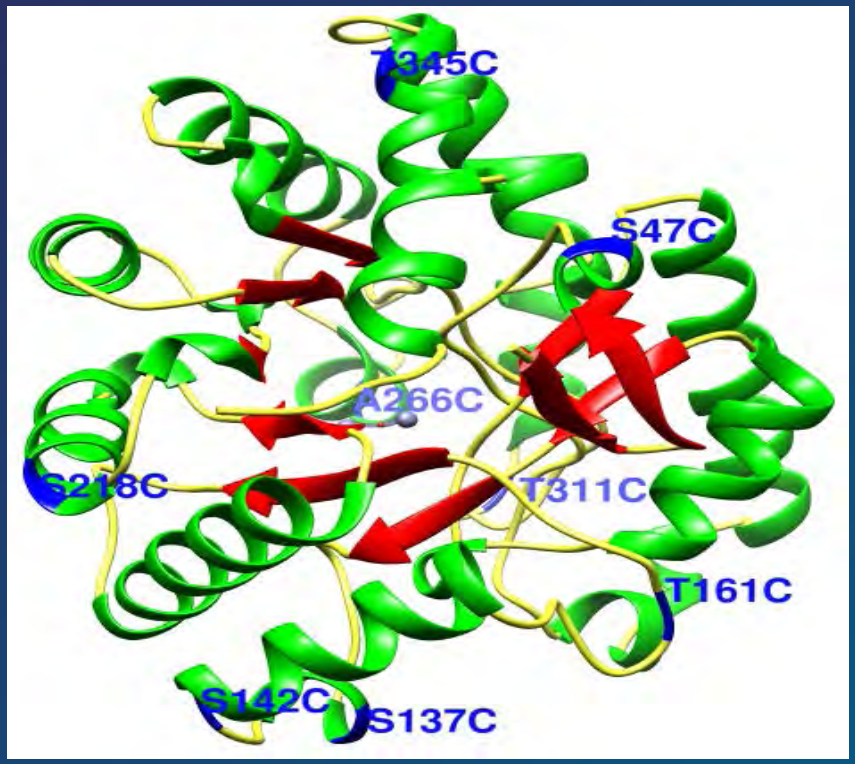


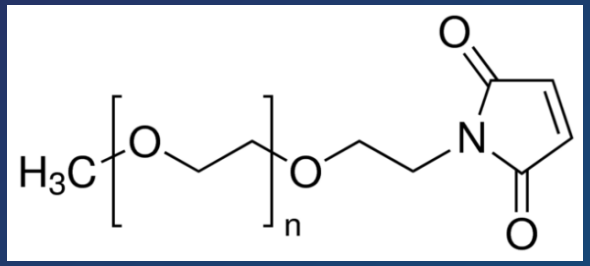
Lipid linkage

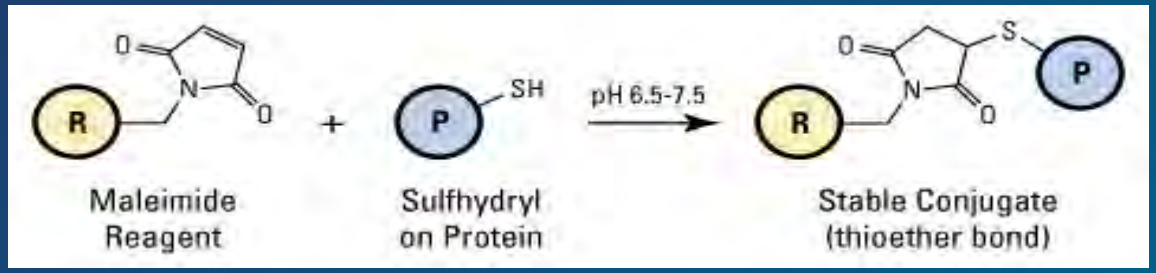
OPHC24S served as control

Topological Analysis of the Lipoprotein Organophosphate Hydrolase (OPH) *Reveals a Periplasmic Localization*









Methoxypolyethylene glycol maleimide

Parthasarathy et al, FEMS Micobiology Letters (2017).

Tat substrates are membrane or extra cellular proteins.

i) **requiring large cofactors**

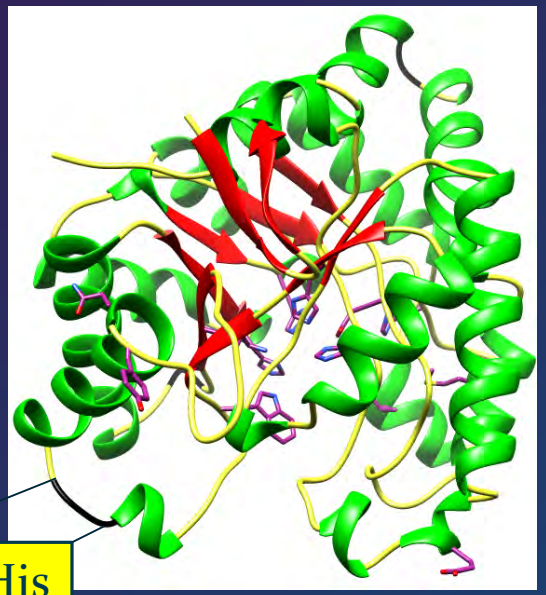
i) **proteins that fail to fold in harsh extra cellular environments?**

i) **Multi-protein complexes**

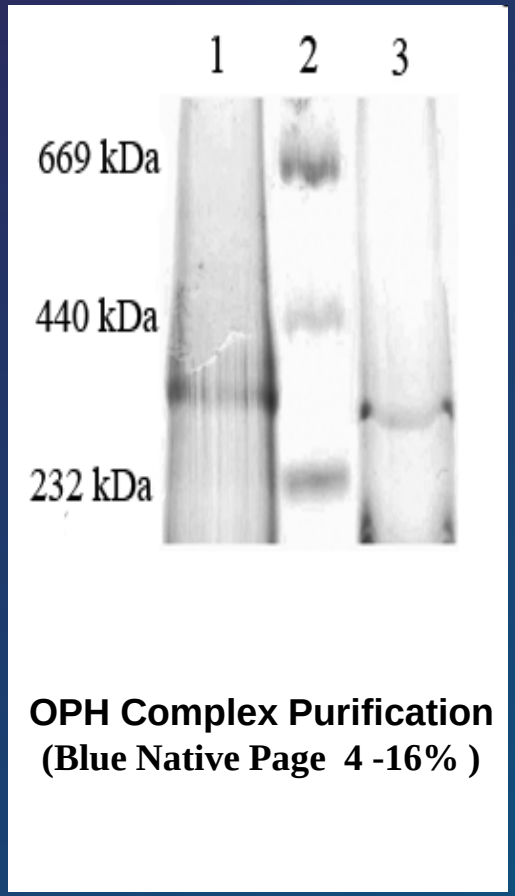
Question: **Why is OPH Tat substrate?**

Purification of OPH Complex from *Sphingophyxis wildii (B. diminuta)*

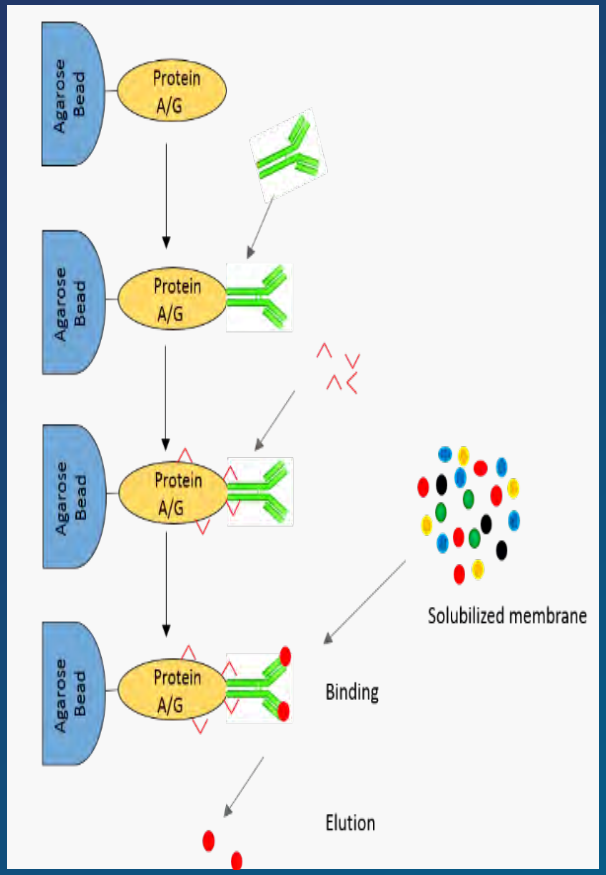
OPH^{14110XHis}



10xHis



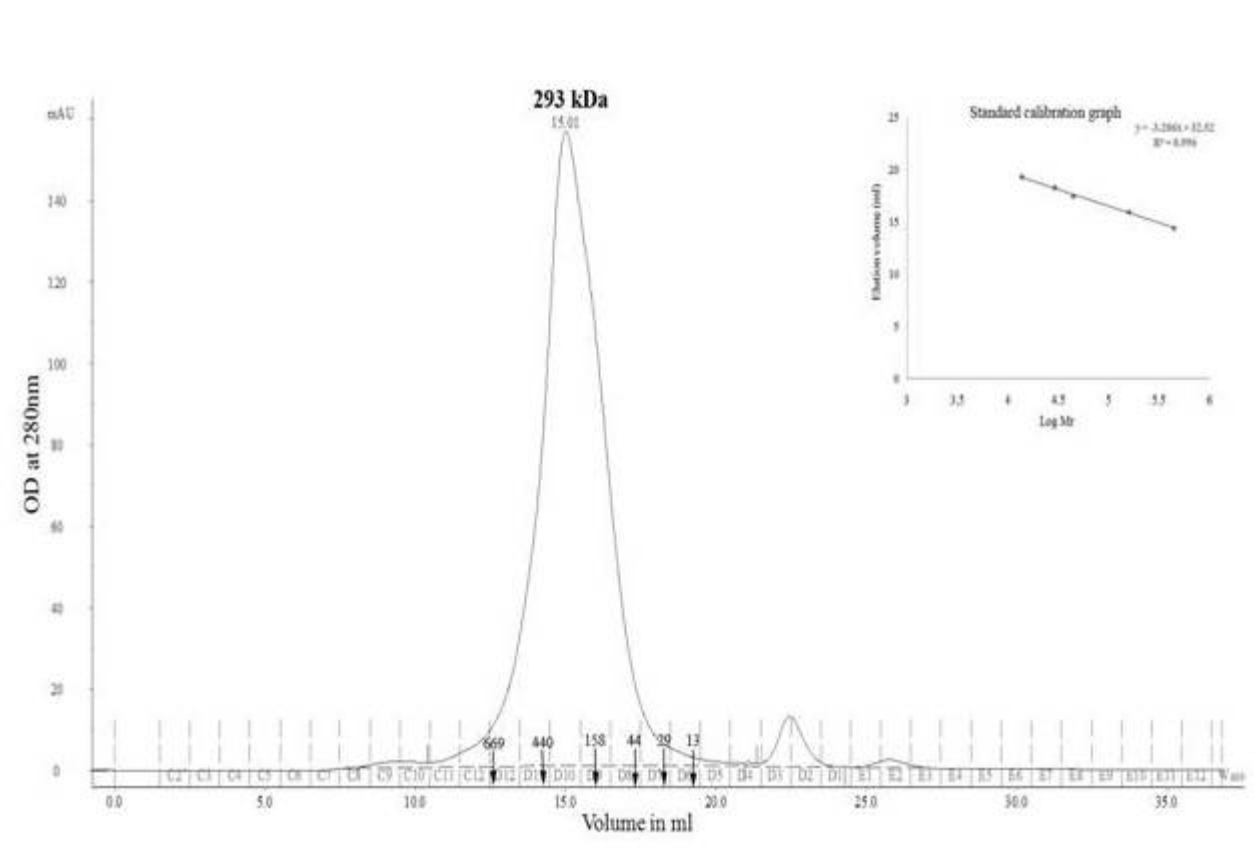
OPH Complex Purification
(Blue Native Page 4 -16%)



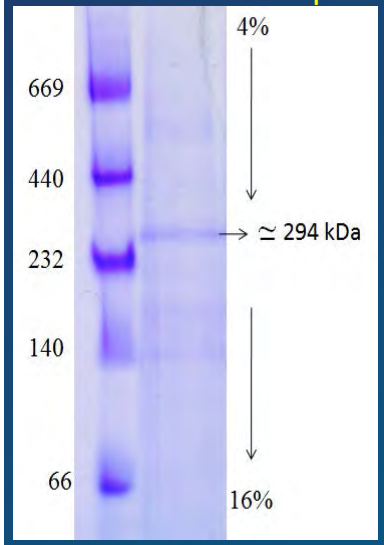
Lane 1 represents Immunopurified OPH complex , Lane 2 represents Molecular weight marker
Lane 3 represents IMAC purified complex

Molecular weight determination of the Purified OPH complex

Gel permeation

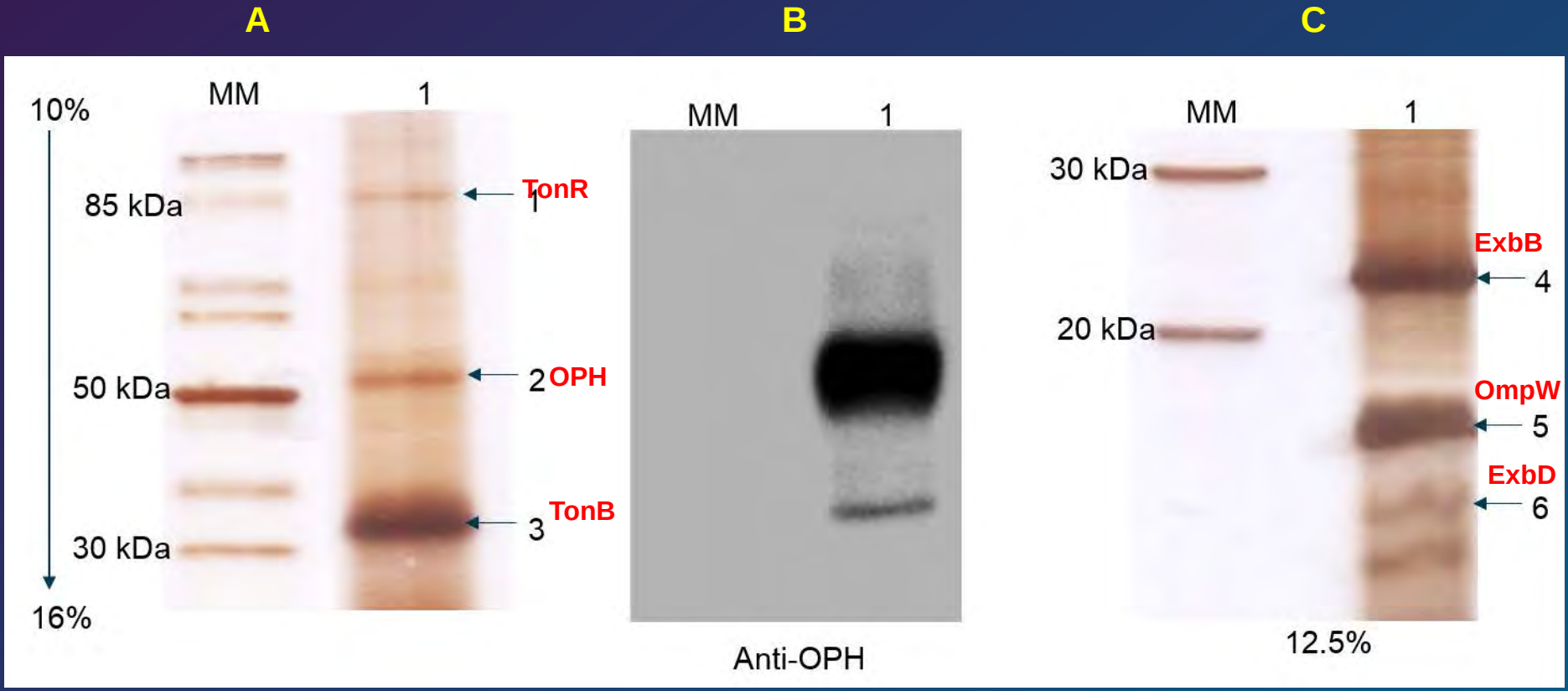


BN PAGE



Parthsarathy et al, (2016) J. Biological. Chem. 291: 7774–85.

TonB dependent Transport Components Co-purify with OPH

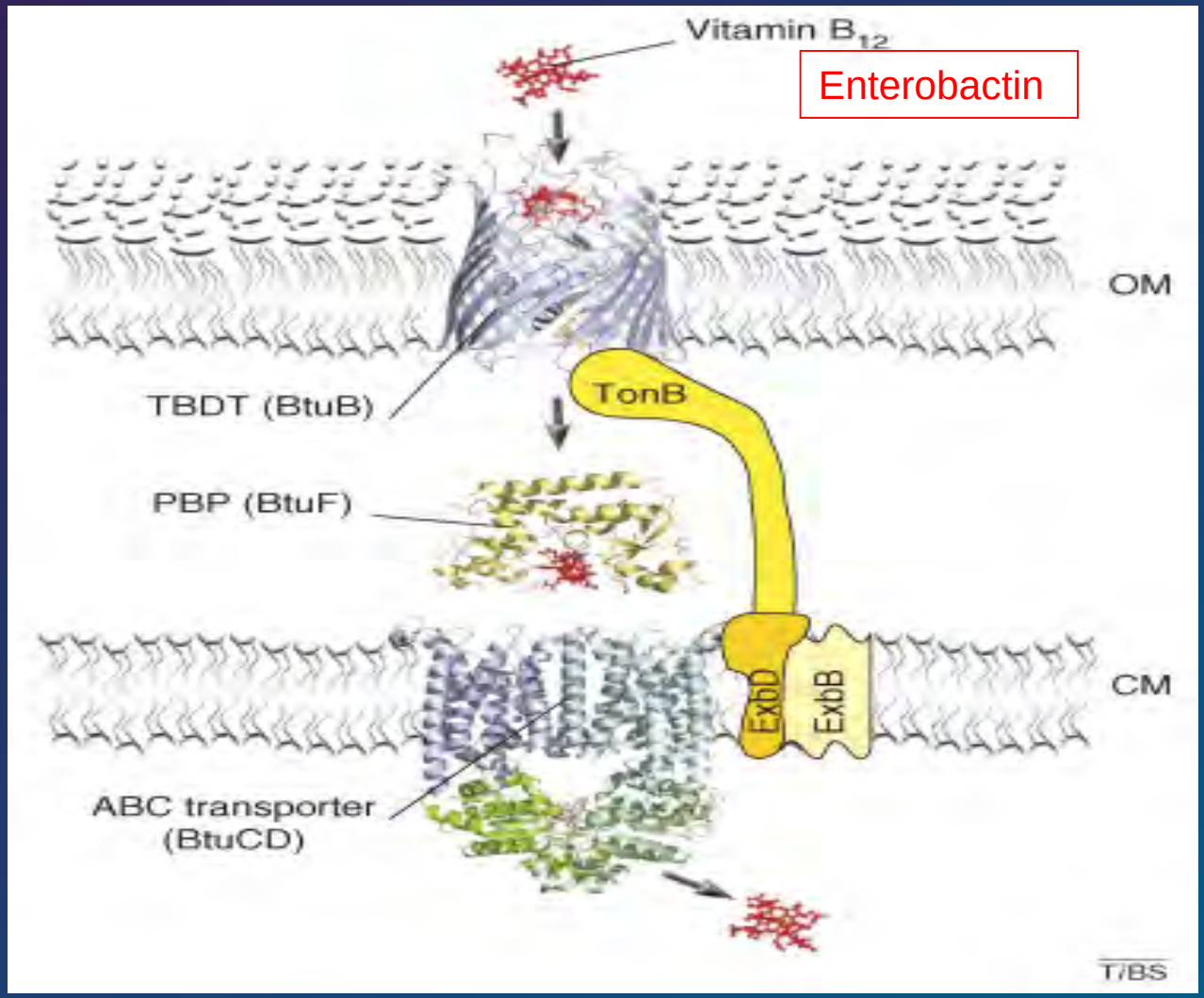


Tricine PAGE profile of proteins co-purified with OPH.

Panels A & C. Profile of OPH-associated proteins obtained on Tricine PAGE. MM molecular weight markers; Lane 1, immune-purified OPH complex. The discrete protein bands resolved on tricine PAGE (Panel A & C)were identified as TonR (1), TonB (3), ExbB (4), Outer membrane porin, OmpW (5) and ExbD (6).

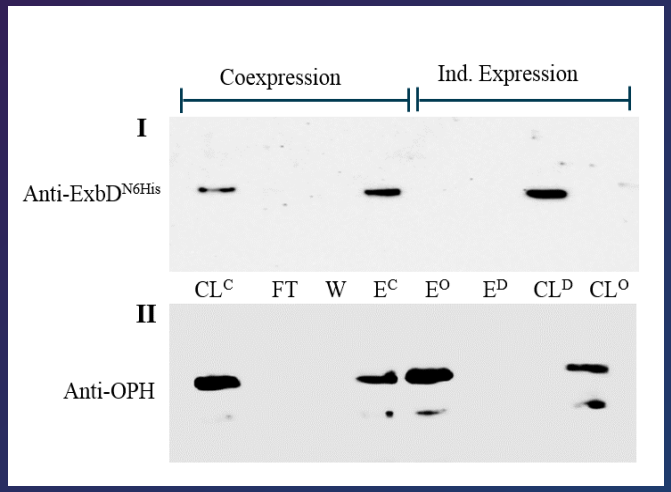
Panel B. The cross-reaction of a 52 kDa protein (2) with OPH-specific antibodies.

TonB Dependent Transport System (TonBDT) In Gram Negative Bacteria

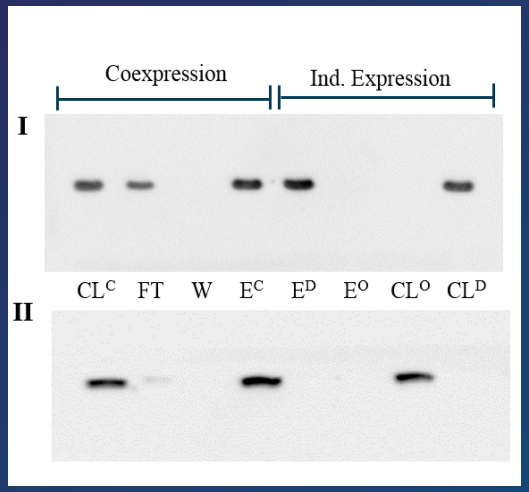


OPH Interacts with ExbD
Reciprocal pull-down & BTH Assays

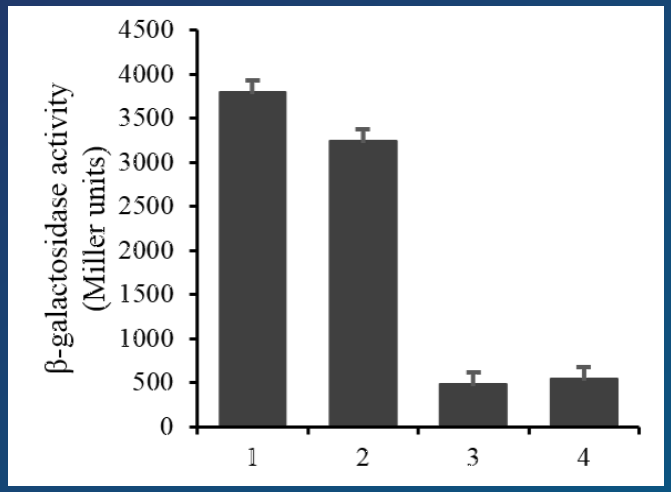
A



B

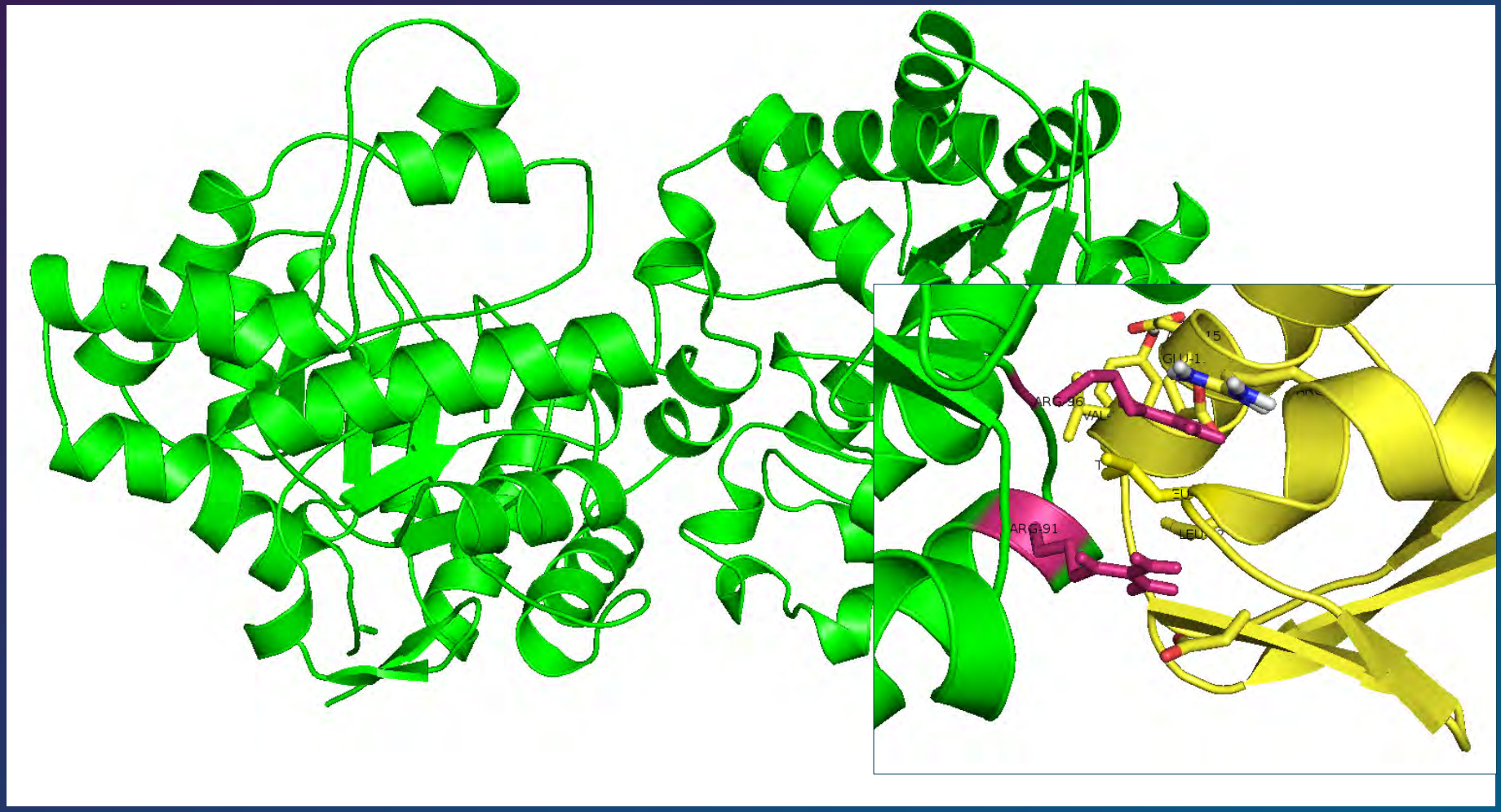


C



Panel A: Proteins co-purified with biotinylated OPH (Streptavidin magnetic beads).
Panel B: Proteins co-purified with ExbD^{NHis} using (Ni-NTA magnetic beads)
CL^{C} represents cell lysate prepared from cells expressing OPH^{CAviTag} and ExbD^{N6His}. Cell lysates having either OPH^{CAviTag} or ExbD^{N6His} are shown as CL^{O} and CL^{D} respectively. FT, W and E indicate flowthrough, wash and elution fractions respectively.
Panel C: β-galactosidase assay ExbD-T18 + T25-OPH (pGS29 +pGS28) (1), OPH-T18 + T25-ExbD(pGS26+pGS31) (2), ExbD-T18 +T25 (pGS29+pKT25) (3) and OPH-T18+ T25 (pGS26+pKT25) (4)

OPH Interacts with ExbD Through Surface Exposed Arginine Residues

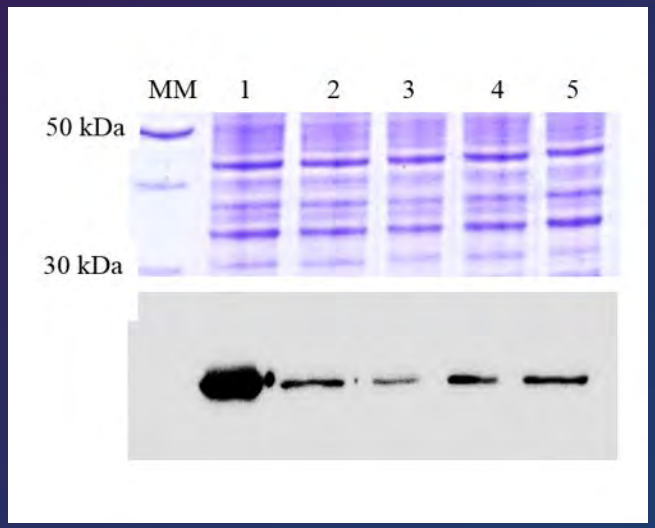


Arg 91
Arg 96

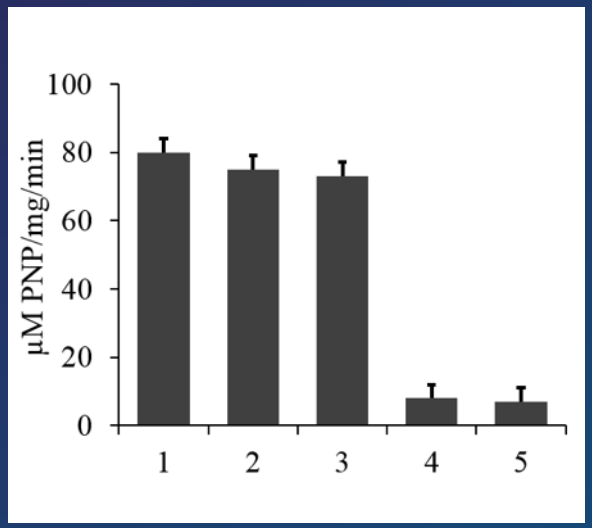
Leu 83
Asp 81

OPH variants $OPH^{R91A,R96G}$ and $OPH^{R91F,R96G}$ Fail to Interact with $ExbD^{N6His}$

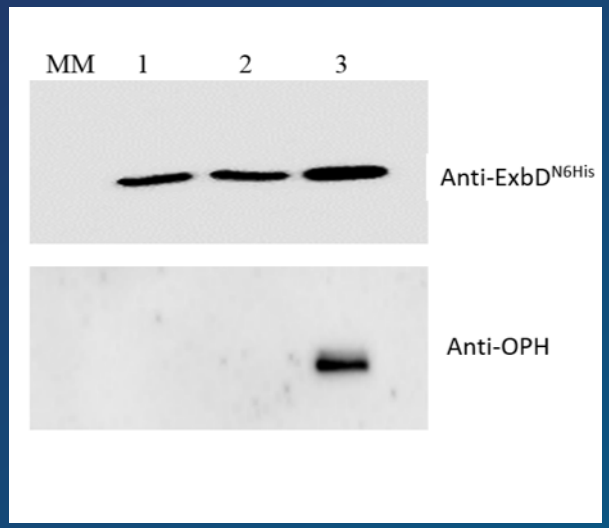
A



B



C



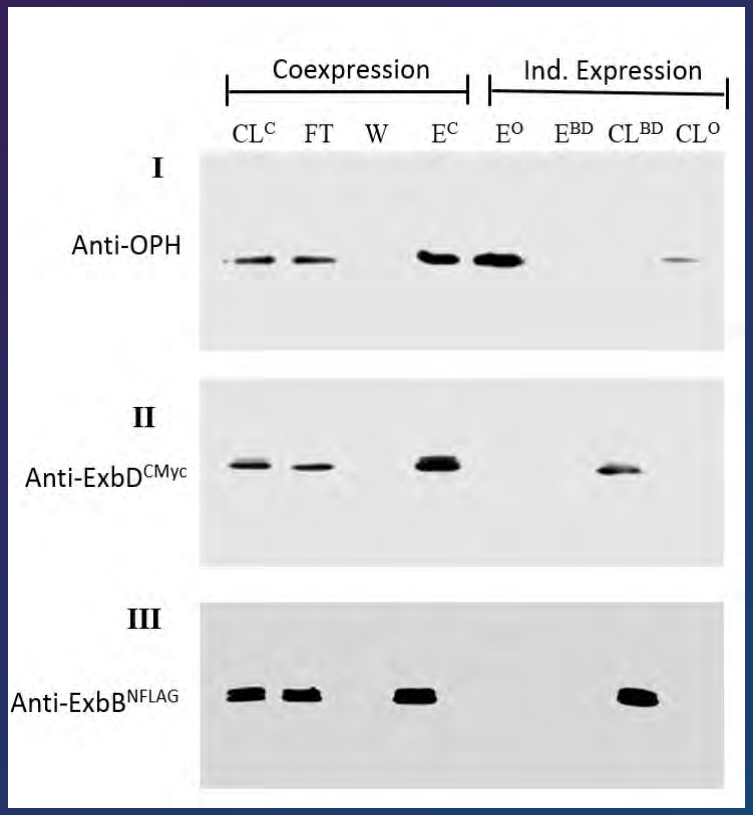
Panel A. SDS-PAGE (12.5%) and corresponding western blots indicating expression, and stability of OPH (lane 1) and its variants $OPH^{R91A,R96G}$ (lane 2), $OPH^{R91F,R96G}$ (lane 3) $OPH^{R91F,\ R96A}$ (lane 4) and $OPH^{R91F,R96F}$ (lane 5

Panel B: The activity of OPH (lane 1) and its variants $OPH^{R91A/R96G}$ (lane 2), $OPH^{R91F/R96G}$ (lane 3), $OPH^{R91F/R96A}$ (lane 4) and $OPH^{R91F/R96F}$ (lane 5)

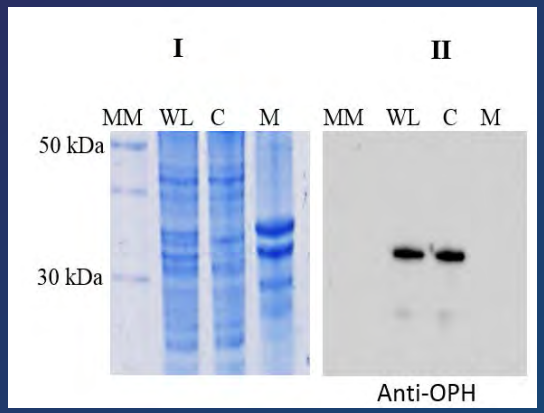
Panel C. Western blot performed to detect proteins found in the elution fractions collected from pulldown assays performed using Ni-NTA magnetic beads and lysate prepared from cells co-expressing $ExbD^{N6His}/OPH^{R91A,R96G}$ (1), $ExbD^{N6His}/OPH^{R91F,R96G}$ (2) or $OPH/ExbD^{N6His}$ (3).

OPH targets membrane only in presence of ExbD And ExbB/ExbD complex

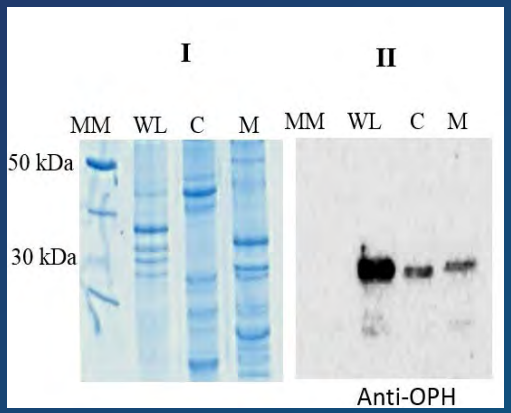
A



B



C



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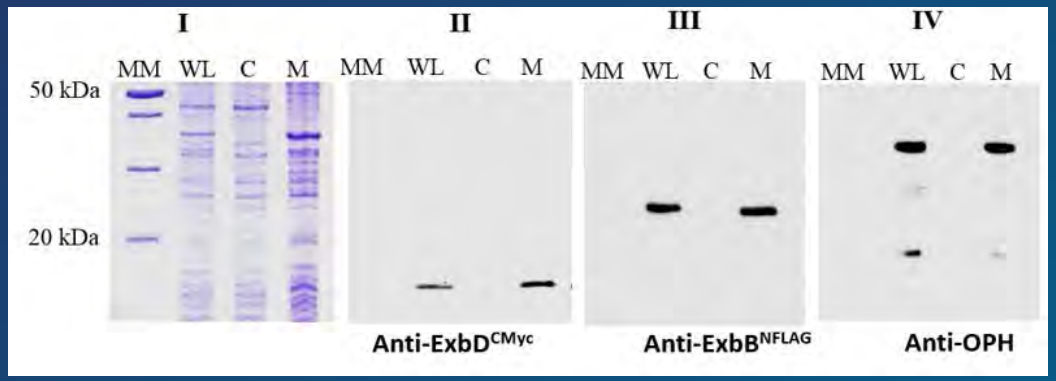
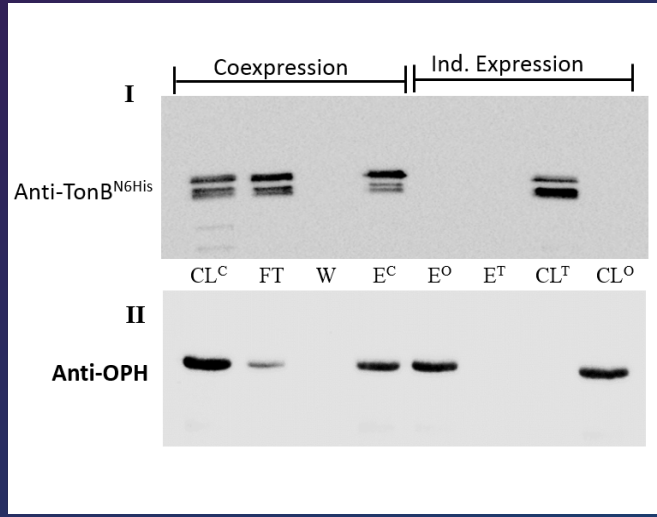


Panel A: **OPH-ExbB/ExbD Interactions. (Pull down with Streptavidin magnetic beads)**
Panel B: Cytosolic localization of OPH
Panel C: Membrane localization of OPH in presence of ExbD^{CMyc}
Panel D: Membrane localization of OPH in presence of ExbB^{NFLAG}/ExbD^{CMyc}

OPH interacts with Energy Transducing Component TonB of TonBDT

(Co-purification of $OPH^{CAviTag}$ and $TonB^{N6His}$).

A



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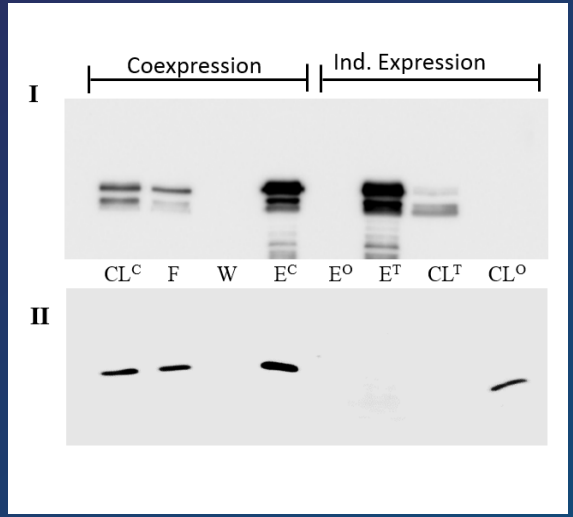

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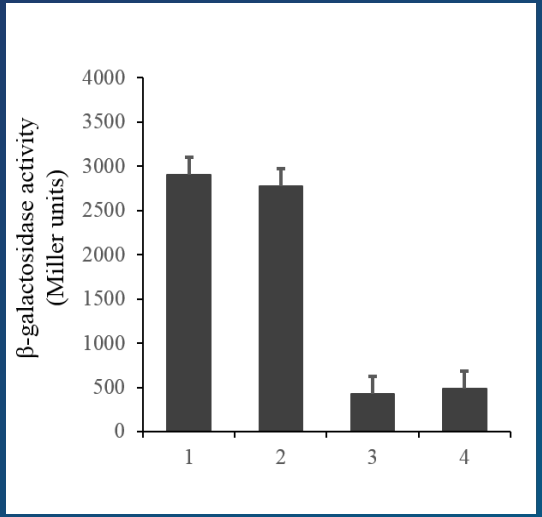
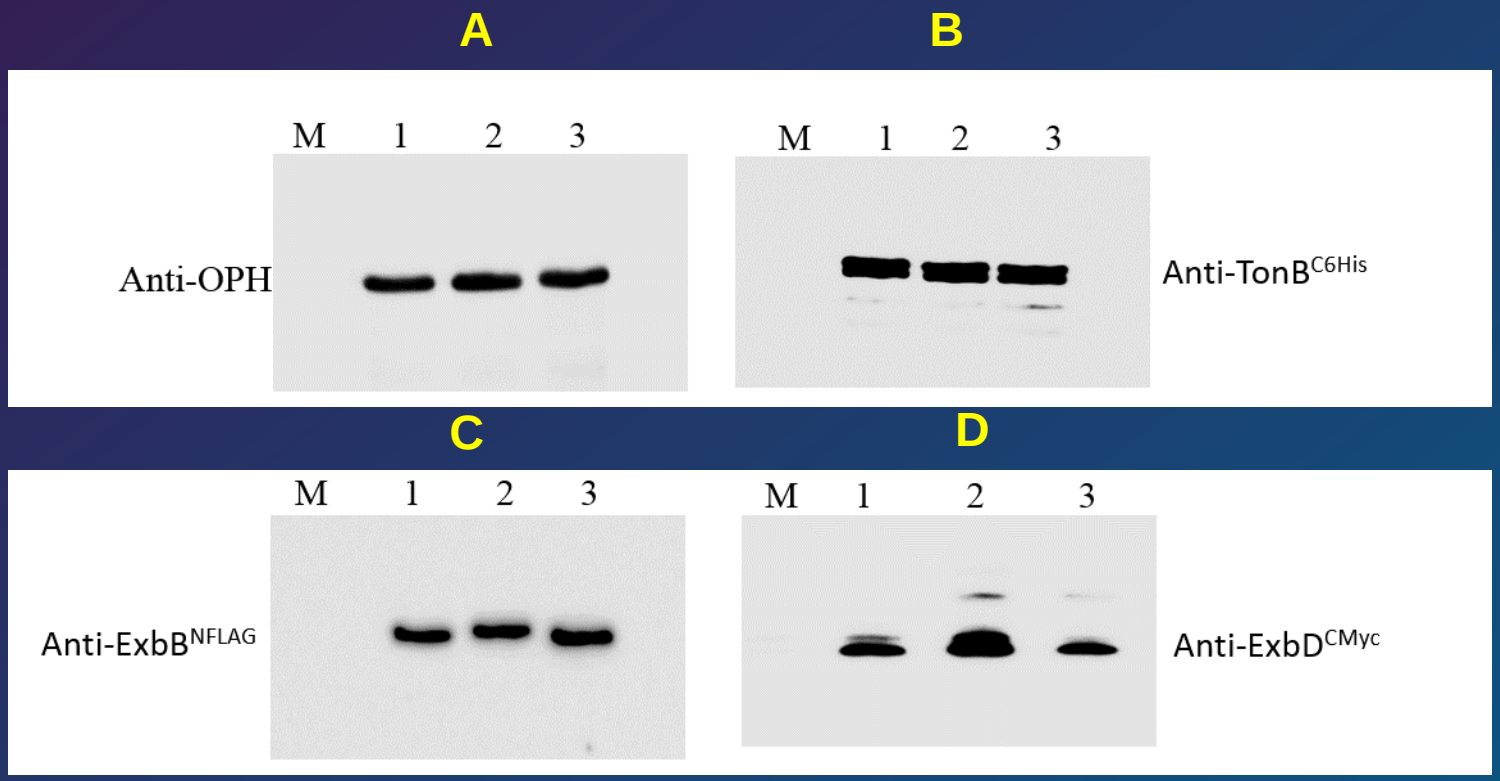


Panel A: Proteins co-purified with biotinylated OPH (Streptavidin magnetic beads)
Panel B: Proteins co-purified with $TonB^{N6His}$ (Ni-NTA magnetic beads)
CL^C represents lysate prepared from cells coexpressing both $OPH^{CAviTag}$ and $TonB^{N6His}$. Lysates having either $OPH^{CAviTag}$ or $TonB^{N6His}$ are shown as CL^O and CL^Trespectively

Panel C: β-galactosidase assay (1) TonB-T18 + T25-OPH (pGS33 + pGS28), (2) OPH-T18 + T25-TonB pGS26+pGS32,(3)TonB-T18 + T25 (pGS33+pKT25) and (4) OPH-T18 + T25 (pGS26+pKT25)

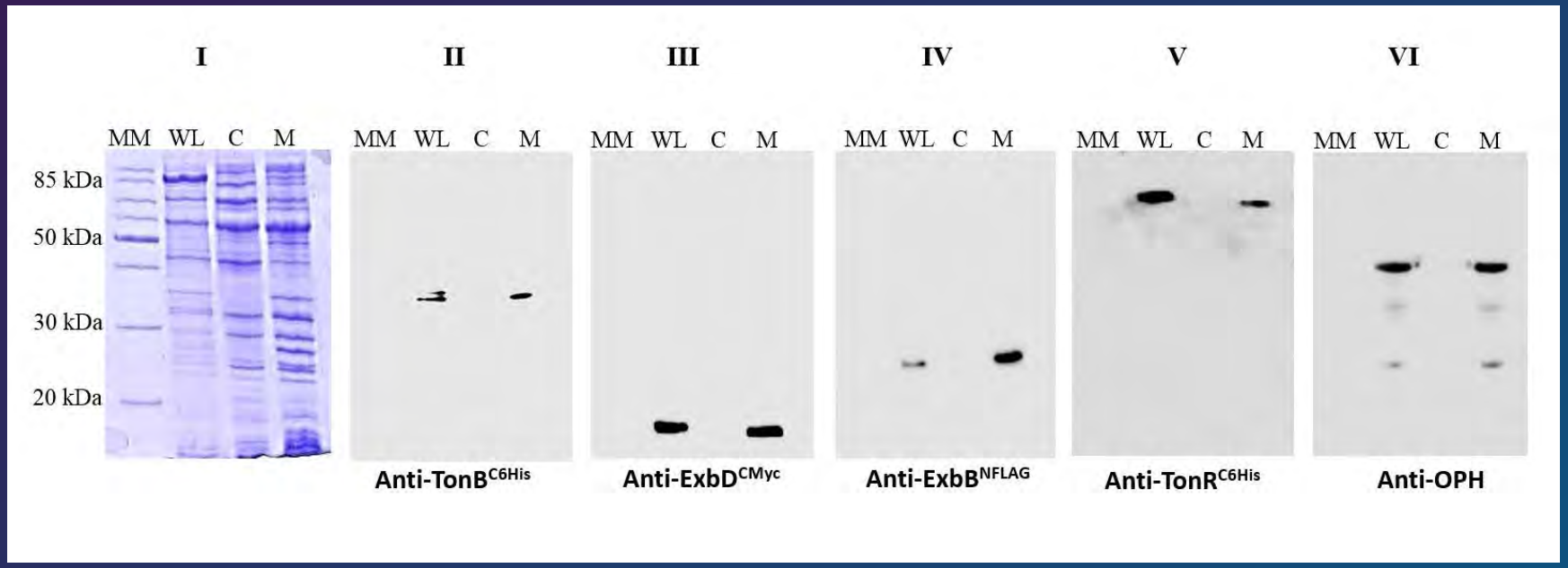
Four component Ton Complex

(Pulldowns with component specific affinity tags)



Panel A, B, C, D: Pull down elution fractions collected from streptavidin magnetic beads (lane 1), Anti-FLAG magnetic beads (lane 2), Ni-NTA magnetic beads (lane 3). incubated with cell lysate of GS027 cells (pGS6+pOPHV400) expressing OPH^{AviTag}, ExbB^{NFLAG}, ExbD^{CMyc} and TonB^{C6His}

Reconstitution of $_{Sf}$TonBDT in *E. coli* GS027.

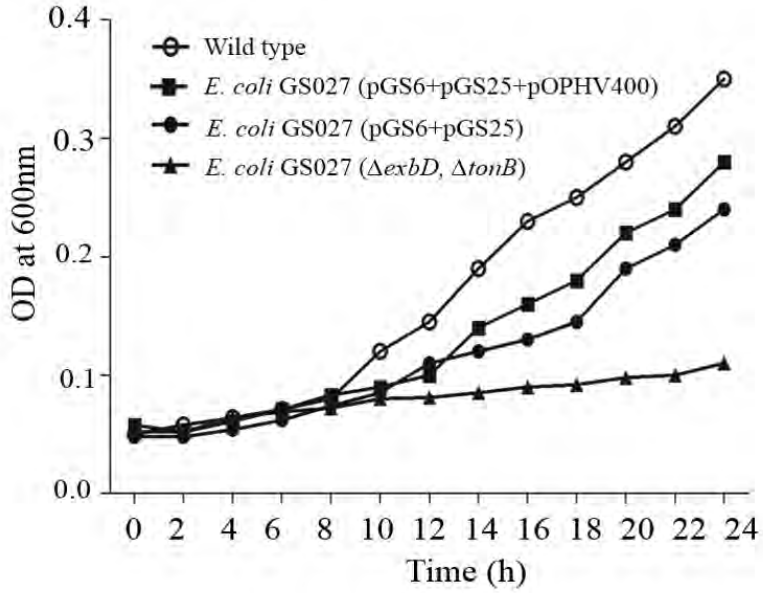


Expression and detection of $_{Sf}$TonBDT components in GS027 cells. GS027 (pGS6+pGS25+pOPHV400) cells were fractionated and the subcellular fractions analyzed on 12. 5% SDS-PAGE (I) was used to probe with anti-His (II), Anti-Myc (III), anti-FLAG (IV) anti-His (V) and anti-OPH (VI) to detect, TonB^{C6His}, ExbD^{CMyc}, ExbB^{NFLAG}, TonR^{C6His} and OPH^{CAviTag} in membrane fractions respectively.

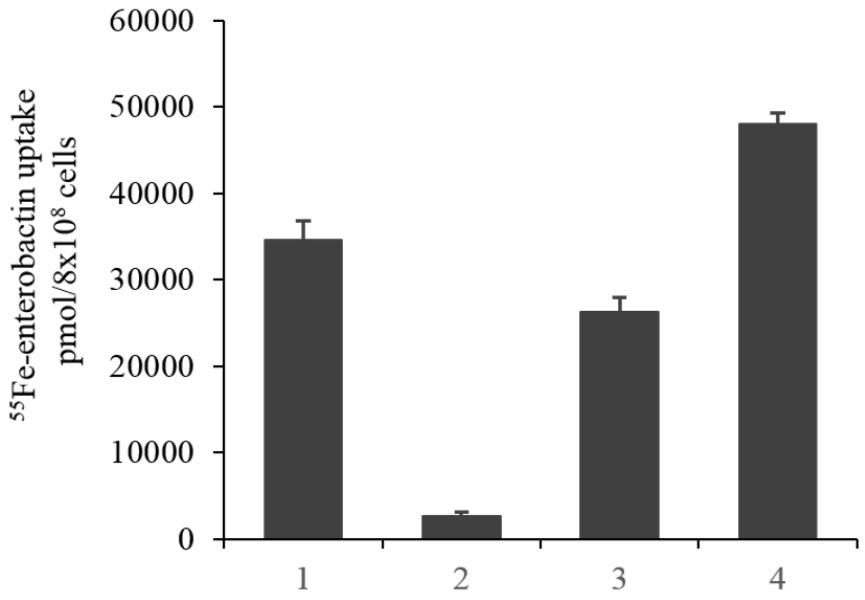
OPH Enhances Iron Uptake

(Four Component Ton Complex is More Efficient Iron Transporter)

A



B

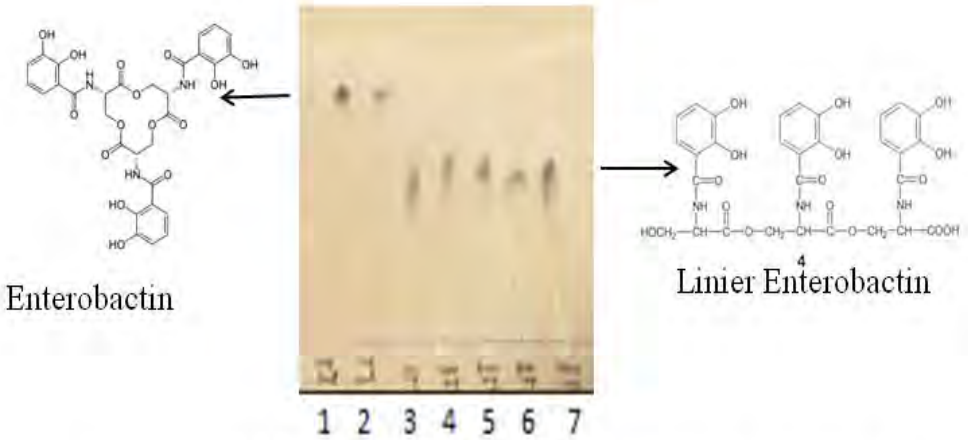


Panel A: Growth of wild type , *E. coli* GS027, and strains complemented with $_{Sf}$TonBDT system without OPH and with OPH under iron-limiting conditions.

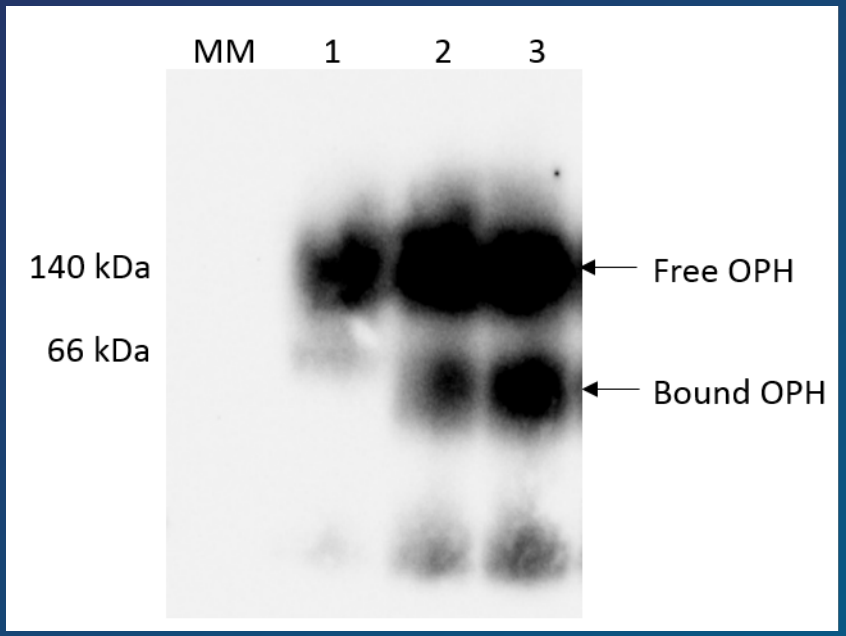
Panel B: ^{55}Fe-enterobactin uptake assay

Lane 1: Wild type Arctic express. Lane 2: GS027, Lane 3: (pGS6+pGS25), lane 4: (pGS6+pGS25+pOPHV400) ExbB^{NFLAG}/ExbD^{CMyc}+TonB^{C6His}+TonR^{C6His}

OPH has Enterobactin Hydrolase Activity

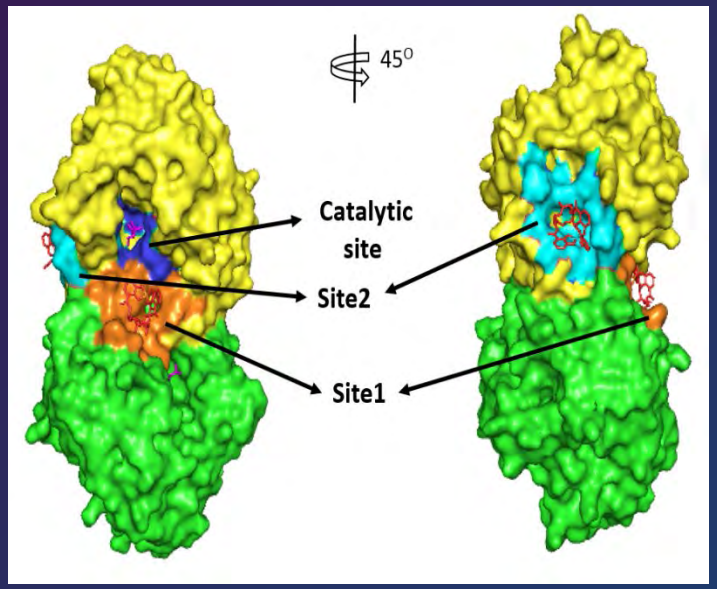


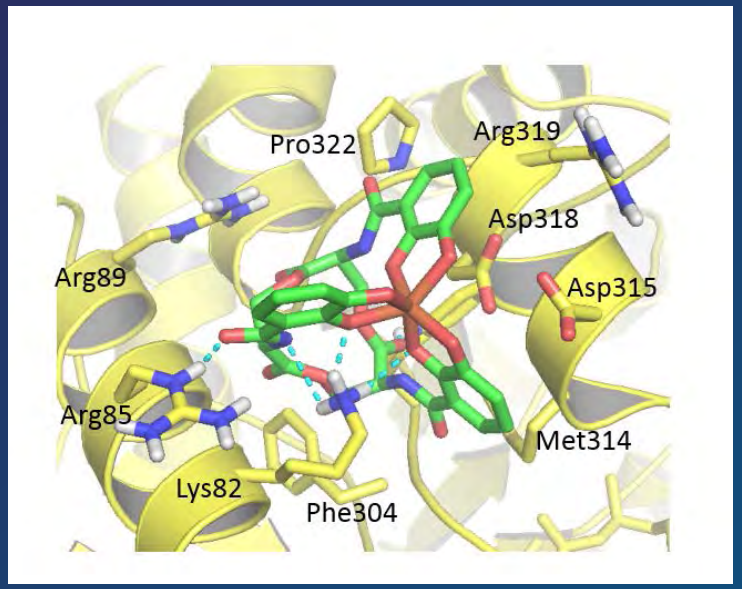
Lanes 1 and 2 Enterobactin, Lanes 3 to 7 represent enterobactin With increased concentration of OPH.



Question: How Does OPH Enhances Iron Uptake.

Enterobactin Binds OPH at the secondary binding site: A Active site lysine is critical for B binding





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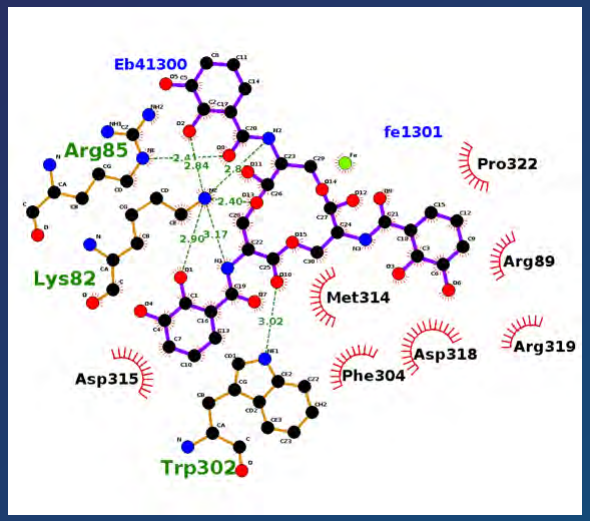

Panel A. *in silico*-predicted enterobactin binding sites Panels B and C. The best pose obtained from the docking study after stabilizing the side chain residues lysine 82 and arginine 85 placed between two of the three catechol rings by hydrogen bonding interactions.

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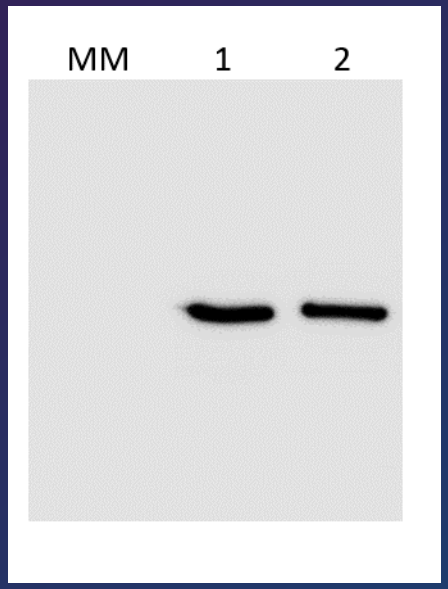
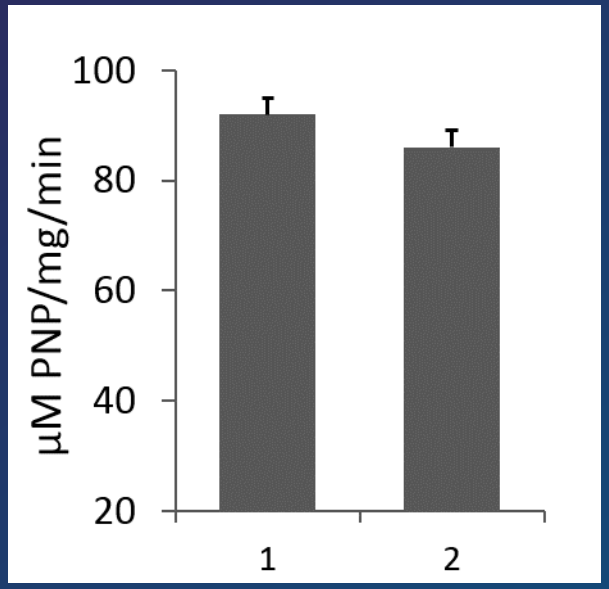
Active site lysine is critical for binding: OPH^{K82A} doesn't bind to Enterobactin

D



E



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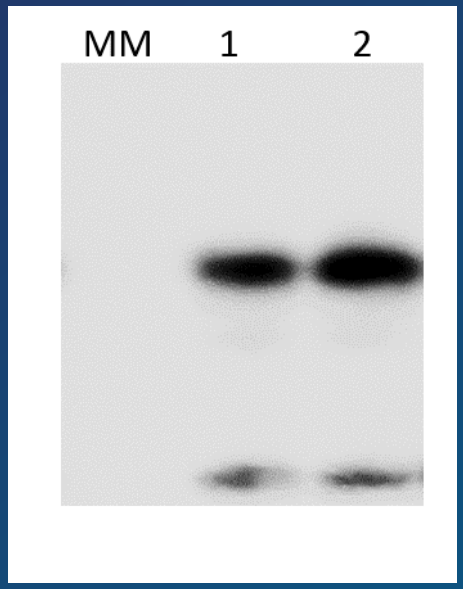
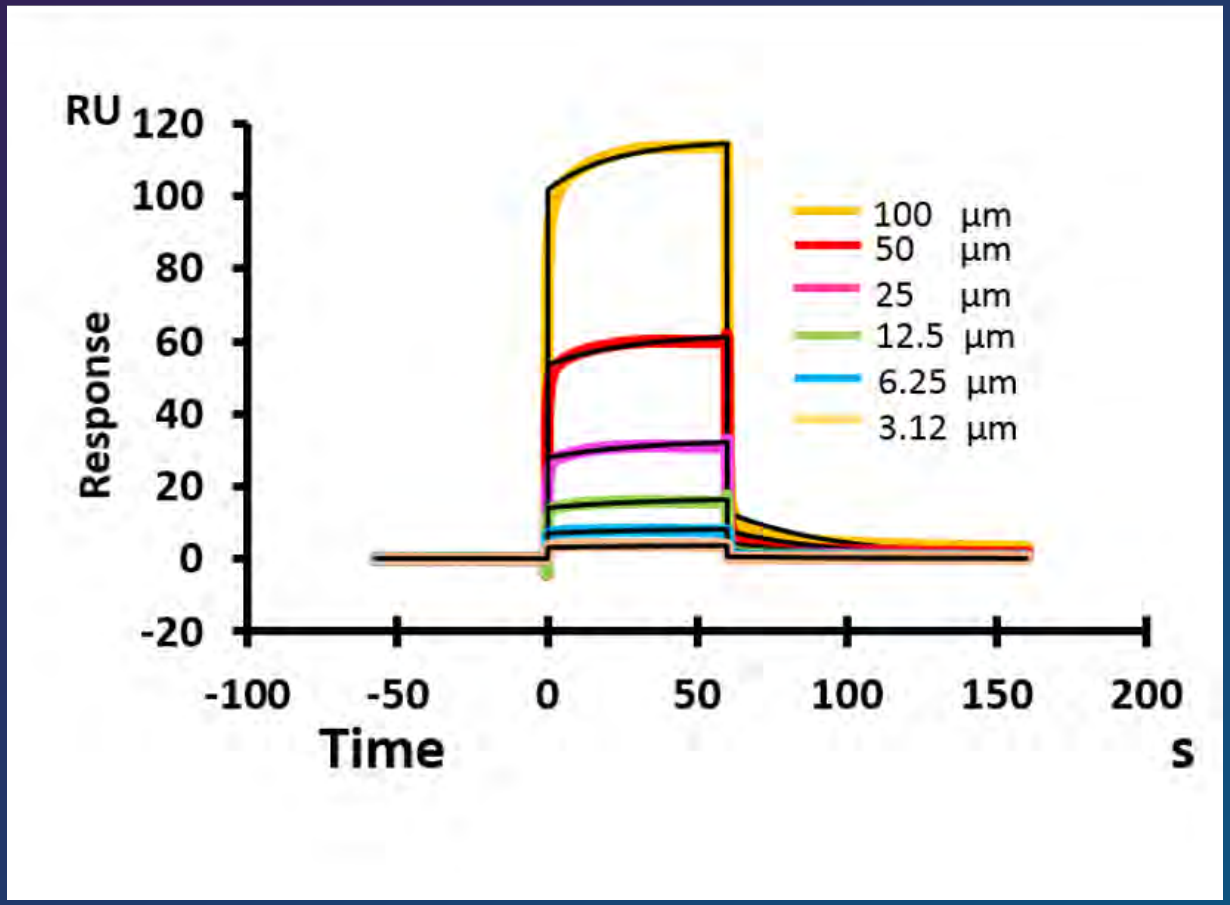


Panels D. The stability of OPH^{K82A}. Proteins extracted from cells expressing OPH (Panel D, lane 1) and OPH^{K82A} (Panel D, lane 2) were analyzed on 12.5% SDS-PAGE and the corresponding western blot was developed using anti-OPH antibody.
Panel E. The activities of native OPH (1) and OPH^{K82A}(2).
Panel-F. The mobility pattern on Native PAGE of OPH^{K82A}incubated without and with Fe-Ent is shown in lanes 1 and 2 respectively.

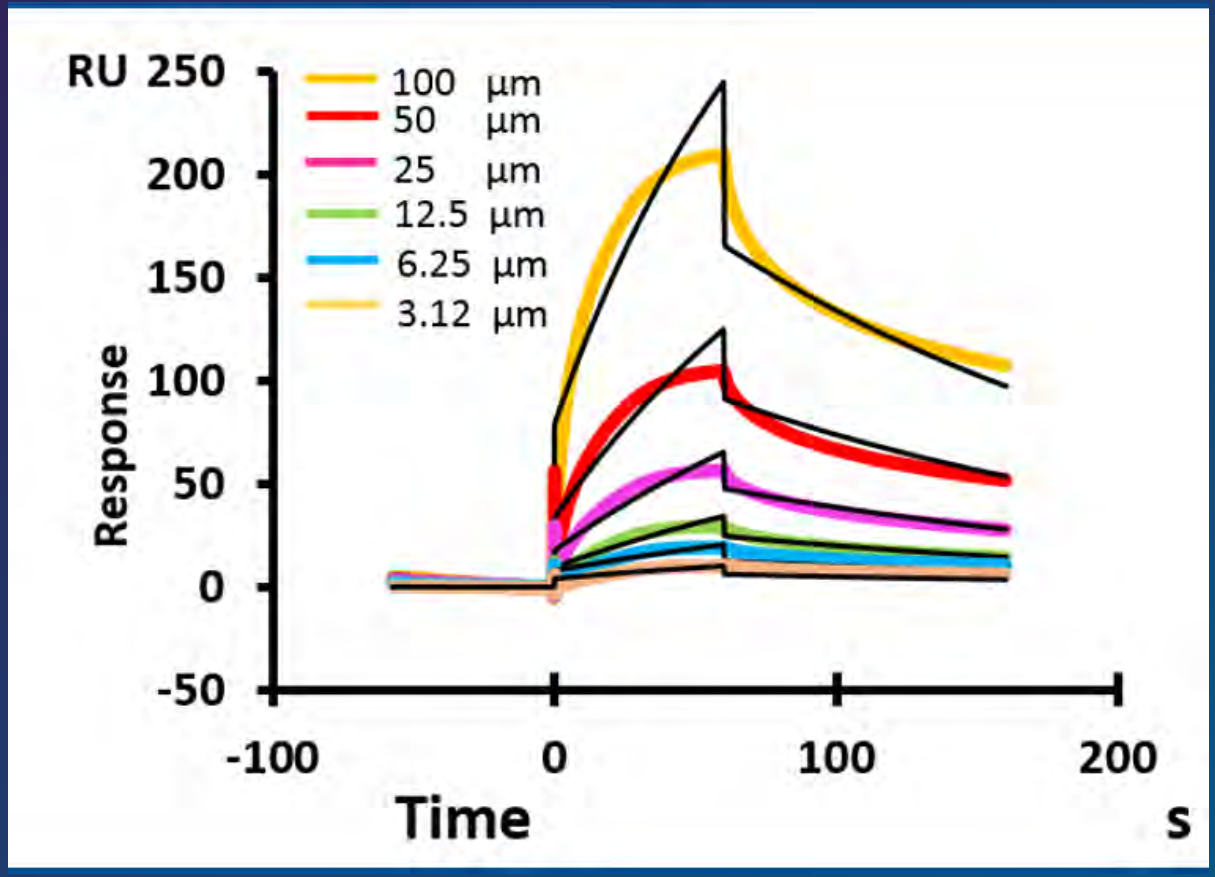
Binding Kinetics
Organophosphate Hydrolase (Ligand) and Enterobactin (Analyte)



Kinetics: Evaluation software 2.0 version
Model 1:1 Binding Model

Sample	KD (M)	SE KD (M)
OPH - Enterobactin	1.24E-04	0.082E-04

Kinetic Analysis
OPH (Ligand) and Ferric Enterobactin [$Fe^{III}(Ent)^{3-}$] (analyte)

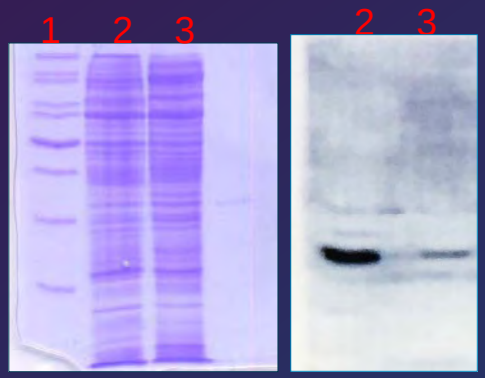


Ligand - OPH
Analyte - $Fe^{III}(Ent)^{3-}$]

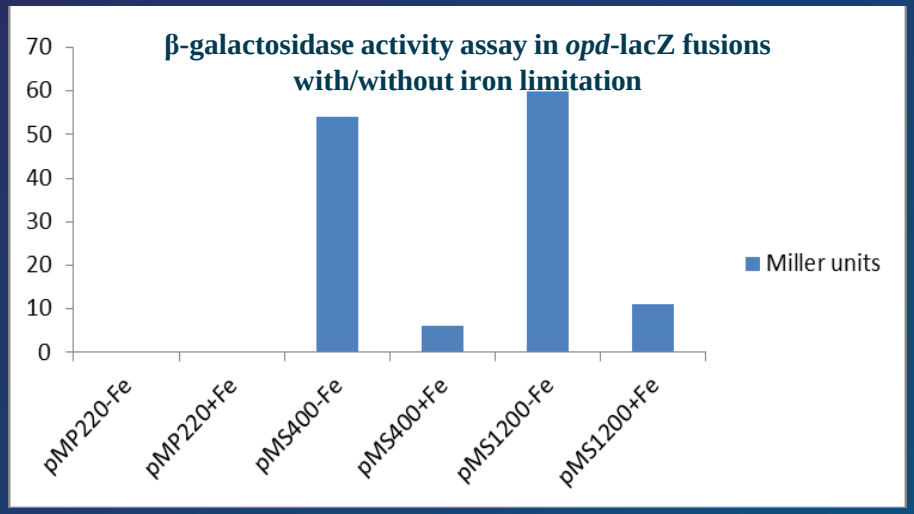
Sample	KD (M)	SE KD (M)
OPH - ferric enterobactin	3.03E-05	0.069E-05

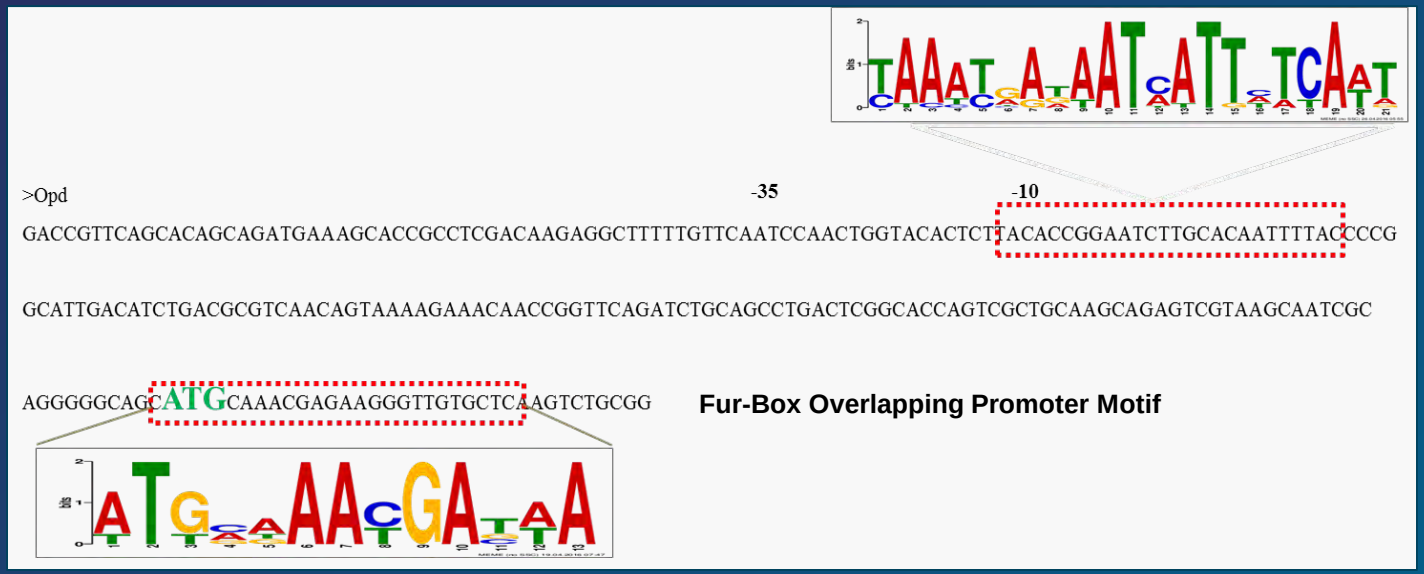
Is OPH Part of Iron Regulon ?

(Expression of OPH in Iron sufficient/ limiting conditions)



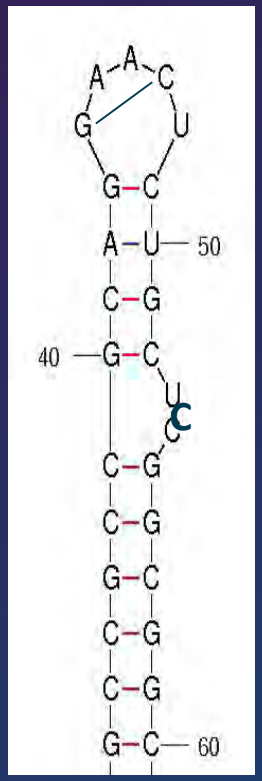
1- Protein ladder
2- wild type *Sphingobium* under iron limiting condition
3- wild type *Sphingobium* under iron sufficient condition



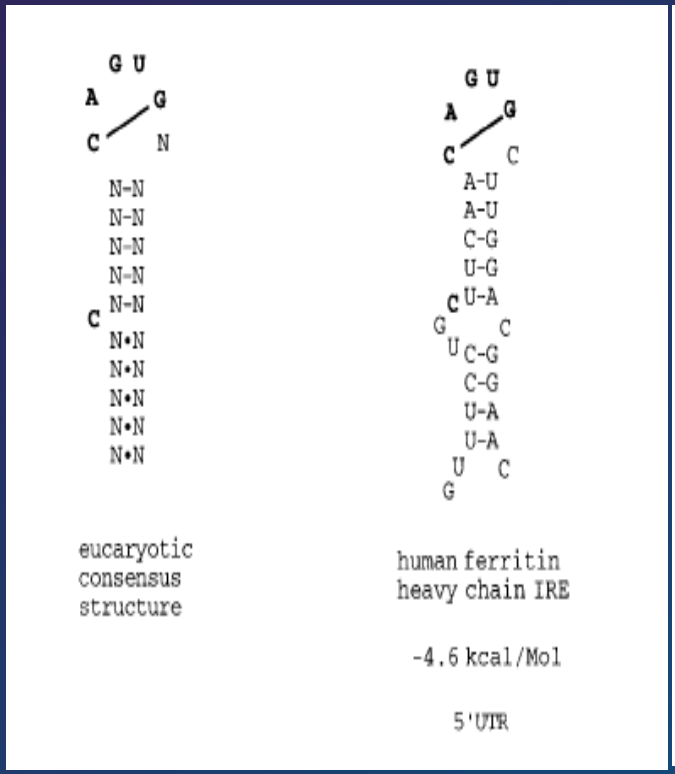


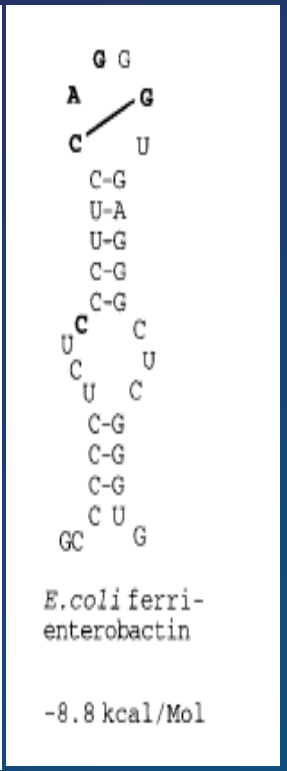
Alignment of *opd* mRNA with well characterized Iron Response Elements

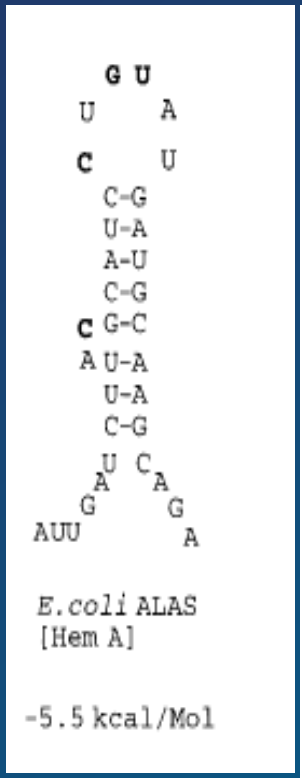
Comparison of *opd* mRNA structure with predicted secondary structures of Iron Response Elements (IREs).

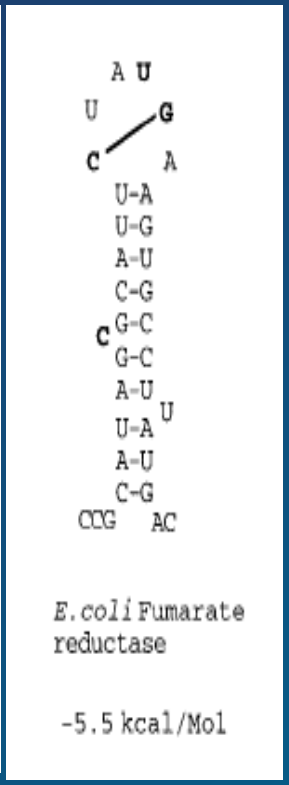


opd mRNA

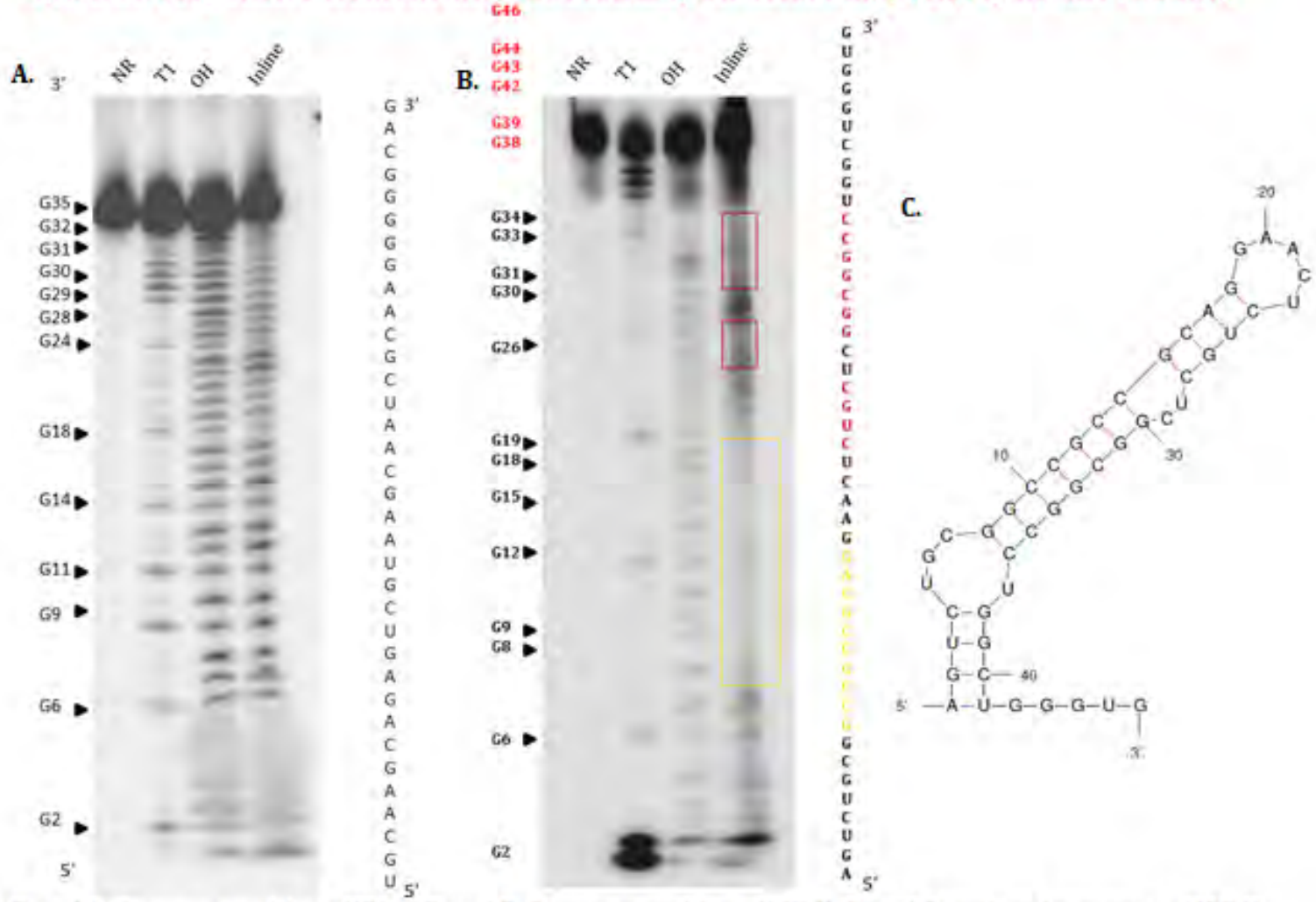






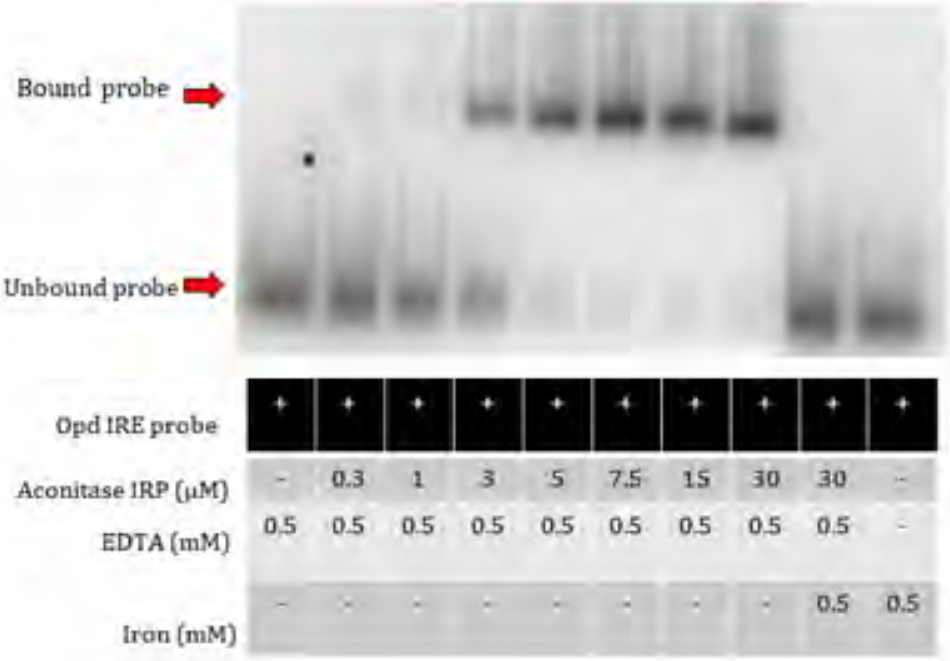


opd Iron responsive element mRNA: secondary structure mapping by In-line probing

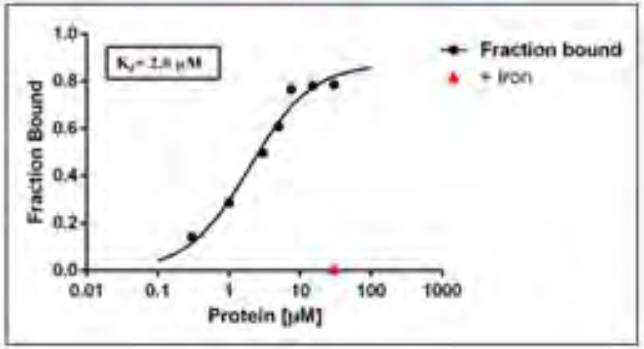


Panel A: Structure probing of IRE-I, **Panel B**: Structure probing of IRE-II, **Panel C**: assigned structure of IRE-II based on in-line results

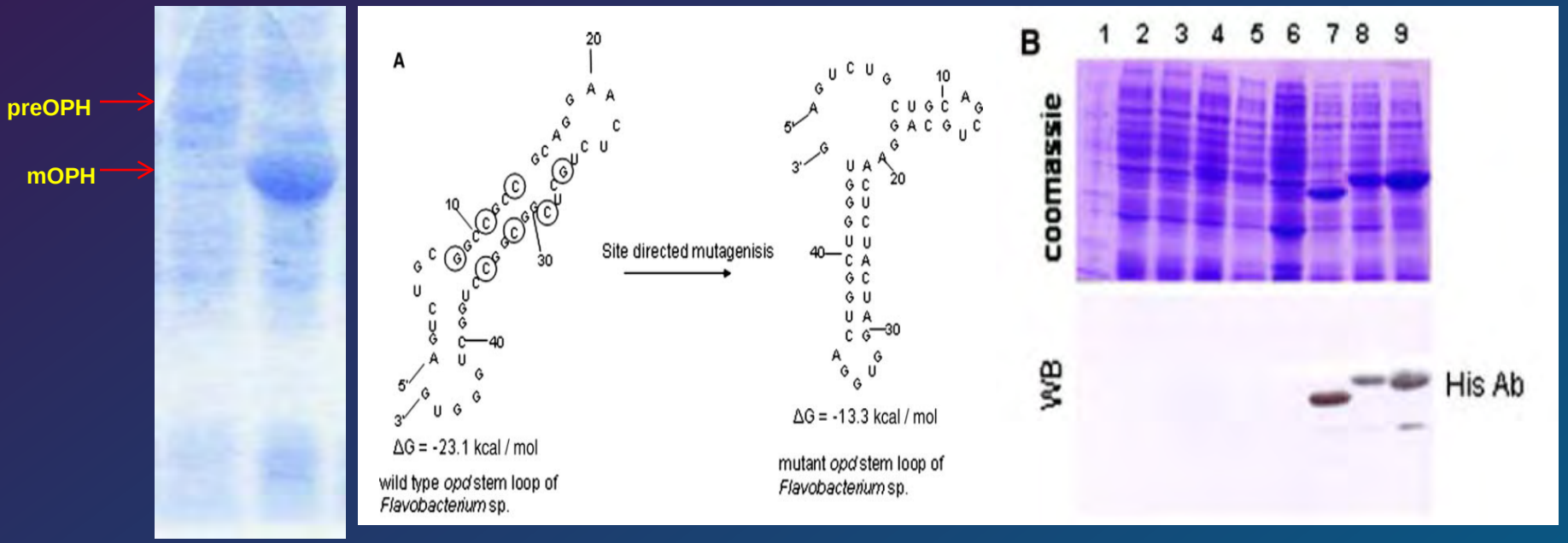
EMSA of *opd* IRE –IRP interaction in the presence/ absence of Iron



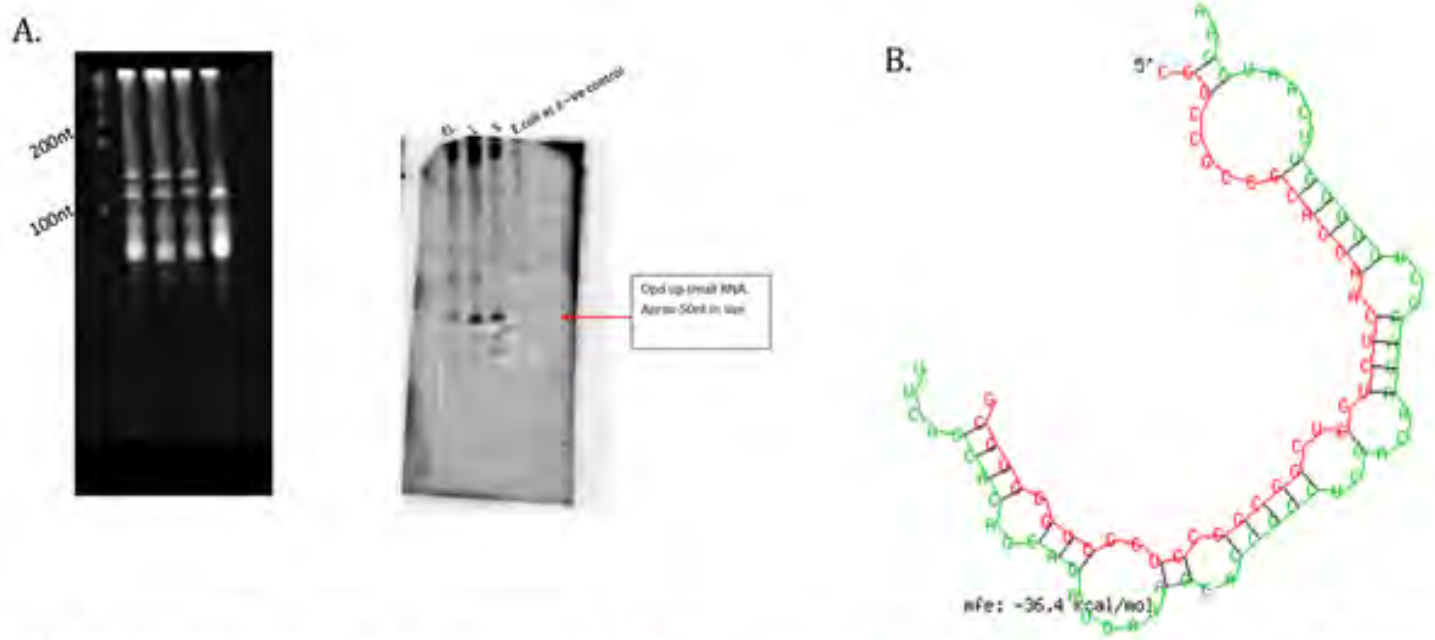
Determination of Dissociation constant



Removal of signal peptide Enhances Expression of OPH in *E. coli*



Represents secondary structures and corresponding ΔG values of wild type *opd* and *opd'* mRNA molecules. Mutations introduced at the third base of the codon in the stem region are indicated with open circles.



Panel A: Northern blot showing the expression of sRNA present upstream of *opd* mRNA (EL indicates Early Log phase, L indicates Log phase, S indicates Stationary phase) of *Sphingobium fuliginis* ATCC27551 cells.

Panel B: Predicted sRNA-mRNA hybrid formed in the region of II-IRE of *opd*.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Dum Biryani
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DST, CSIR, DBT & DRDO
Biological E Pvt Ltd.,
Aurabindo Pharma