Organophosphate hydrolase interacts with ferric-enterobactin and promotes iron uptake in association with TonB dependent transport system.

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ABSTRACT

Our previous studies have shown the existence of organophosphate hydrolase (OPH) as a part of the inner membrane associated TonB complex (ExbB/ExbD and TonB) of Sphingobium fuliginis. We now show its involvement in iron uptake by establishing direct interactions with ferric-enterobactin. The interactions between OPH and ferric-enterobactin were not affected even when the active site architecture is altered by substituting active site aspartate with either alanine or asparagine. Protein docking studies further substantiated these findings and predicted the existence of ferric-enterobactin binding site that is different from the catalytic site of OPH. A lysine residue (82 K) found at the predicted ferric-enterobactin binding site facilitated interactions between OPH and ferric-enterobactin. Substitution of lysine with alanine did not affect triesterase activity, but it abrogated OPH ability to interact with both ferric-enterobactin and ExbD, strengthening further the fact that the catalytic site is not the site for binding of these ligands. In the absence of interactions between OPHK82A and ExbD, OPHK82A failed to target membrane in E. coli cells. The Sphingobium fuliginis TonB dependent transport (SfTonBDT) system was reconstituted in E. coli GS027 cells generated by deleting the exbD and tonB genes. The E. coli GS030 cells having SfTonBDT system with OPH showed increased iron uptake. Such an increase was not seen in E. coli GS029, cells having SfTonBDT system generated either by omitting OPH or by including its variants, OPHD301A, OPHD301N suggesting a role for OPH in enhanced iron uptake.
INTRODUCTION

Phosphotriesterases (PTEs), also known as organophosphate hydrolases (OPH), are present in a number of soil bacteria. These binuclear metallo-enzymes hydrolyze P-O, P-S and P-C bonds found in a variety of organophosphate insecticides and nerve agents (1). The inner membrane associated OPH contains a 23 amino acid long signal peptide with unique characteristic features. It contains a Twin Arginine Transport (TAT) motif (MQTRRVVLK) at the N-terminus and a lipobox motif (LAGC) at the signal peptidase cleavage site (2). OPH is targeted to the inner membrane in a prefolded conformation and remains anchored to it through a diacyl glycerol moiety linked to an invariant cysteine residue present at the junction of signal peptidase cleavage site (2, 3). There are no transmembrane domains in OPH and the entire protein exists in the periplasmic space (3). Recent studies have shown the existence of OPH as part of a multiprotein complex (3, 4). The TonB dependent transport system components, TonB, ExbB/ExbD were co-eluted when the membrane associated OPH was affinity purified from either *S. fuliginis* or *S. wildii* (2, 3). The OPH specifically interacts with the inner membrane associated proton motif force component ExbD and energy transducer TonB to form a four component-TonB complex (4). OPH is targeted to the inner membrane only when co-expressed with ExbB/ExbD. In their absence it remains in the cytoplasm suggesting that formation of a multi-protein complex in the cytoplasm is essential for OPH before it targets the membrane.

TonB dependent transport system plays a key role in the transport of nutrients across the energy deprived outer membrane (5). It contains an outer membrane transporter also known as TonB dependent transporter (TBDT) and an inner membrane associated TonB complex comprising of an energy generating proton motive force (PMF) components ExbB/ExbD and energy transducer TonB. Upon binding to the substrate, the TBDT
undergoes conformational change exposing a pentapeptide motif (ETIV), known as TonB box. The inner membrane associated TonB protein specifically interacts with TonB box and transduces energy harvested from PMF components ExbB/ExbD (6). Initially this unique outer membrane transport system was identified with the infection of phage T1 (7). Soon its role was established in the transport of ferric-enterobactin, vitamin B12 and a number of other nutrients in both pathogenic and non-pathogenic Gram negative bacteria (5).

Lateral transfer of OPH coding organophosphate degradation (opd) genes among soil bacteria is a known phenomenon (8, 9). Identical opd genes, identified as part of mobile elements or self-transmissible plasmids, code for OPH in taxonomically unrelated bacteria (10). A strong selection pressure, accumulated in agricultural soils due to repeated and indiscriminate use of chemicals, is believed to have a role in evolution and lateral transfer of opd genes (11, 12).

In view of OPH association with TonBDT and inherent relationship with lactonases, we have examined if it has a role in transport of ferric-enterobactin. Our investigations failed to show any enterobactin hydrolase activity to OPH. However, we found existence of high affinity interactions between OPH and Ferric-enterobactin. Further, the wild type Sphingopyxis wildii cells showed better iron uptake and growth over opd negative mutants. Similar effect was seen in E. coli GS029 cells reconstituted with OPH containing SfTonBDT system suggesting a role for OPH in iron uptake.

**METHODOLOGY**

**Strains, plasmids and growth conditions**

Bacterial strains and plasmids used in this study are listed in table 1. All biochemicals, including desferri-enterobactin (Ent) were procured from Sigma Aldrich, India. Restriction and other enzymes used in gene manipulations were procured from Fermentas, India. The
basic gene manipulation techniques were performed essentially by following standard procedures described elsewhere (13). The *E. coli* and *Sphingopyxis wildii* strains were grown at 37°C and 30°C respectively either in LB or in minimal medium (3). Expression of *s*TonBDT system components were induced in mid log phase grown *E. coli* Arctic Express cells at 18°C. The iron-limiting minimal medium was prepared following procedures described elsewhere (14). When necessary, antibiotics ampicillin (100μg/ml), kanamycin (30μg/ml), polymyxinB (10μg/ml), tetracycline (20μg/ml) or chloromphenicol (20μg/ml) were supplemented to the growth medium. Oligonucleotides used in this study are listed in supplementary table 1.

**Enzyme assays**

The OPH activity was determined by following standard procedures established in our laboratory (15). Cell fractionation and marker enzyme assays were performed following procedures described elsewhere (2). Nitrate reductase, and glucose-6-phosphate dehydrogenase were used as membrane and cytoplasmic marker enzymes. The purity of membrane and cytoplasmic fractions were established by assaying maker enzymes in subcellular fractions (2). The enterobactin hydrolase assay was performed by following protocols described elsewhere using ferric-enterobactin as substrate (16). Briefly the stock solution was prepared by dissolving 1mg of ferric-enterobactin in 100μl of DMSO and was used to dispense 1mM of ferric-enterobactin into prechilled sterile eppendorf tubes. The test reaction contained pure OPH (1μg) and the control reaction was prepared without adding OPH. The volume of the reaction was then adjusted to 6μl with 50mM sodium phosphate buffer pH 8.0 before incubating the tube at 37°C for 15minutes. After incubation the reaction mix was extracted with ethyl acetate and analyzed on TLC plate to detect formation of linear enterobactin (16).
**OPH interactions with desferri-enterobactin and ferric-enterobactin.**

The OPH interactions with desferri-enterobactin and ferric-enterobactin were studied by performing native PAGE, surface plasmon resonance and fluorescence emission measurement using purified mature form of OPH (OPH) and commercially procured desferri-enterobactin. OPH was purified from *E. coli* (pUCOPH) cultures grown in 5 liters of terrific broth by following established protocols (17, 18).

**Detection of OPH-Ent Complex**

Native PAGE was performed to detect interactions between OPH and desferri-enterobactin/ferric-enterobactin. Initially 10μM of OPH was mixed either with 100μM of ferric-enterobactin \[\text{Fe}^{III}(\text{Ent})^3-\] or with 100μM of desferri-enterobactin and incubated at room temperature for 10 to 20 minutes. After incubation the samples were mixed with native PAGE sample buffer (4x) (50mM Bis-Tris, 6 N HCl, 50mM NaCl, Glycerol (10%), Ponceau S (0.001%), pH 7.2) and loaded, along with native molecular weight markers (GE Healthcare, India), on Novex 4-16% Bis-Tris precast gels (Invitrogen, USA) and electrophoresis was performed following essentially the manufacturers protocols. After electrophoresis the gels were destained and the western blots were performed using anti-OPH antibodies following established procedures (3).

**Surface plasmon resonance (SPR)**

The SPR analysis was used to study OPH interactions with desferri-enterobactin and ferric-enterobactin. The pure OPH was covalently immobilized by amine coupling on a carboxymethylated dextran sensor chip CM7 (GE Healthcare) (19). The amine coupling was done by taking 50μg/ml of OPH protein as ligand in 10mM sodium acetate buffer (pH 5.0). The ligand was then injected at a flow rate of 30μl/min with a contact time of 60 seconds. The process was continued until it captured ~5340 response units (RU). The blank (reference) surface was treated in a similar manner using the same buffer prepared by...
omitting OPH. Ferric-enterobactin was injected as analyte in a running buffer (PBS, pH 7.4 + 0.005% P20 + DMSO 2%). Buffer optimization was done to correct variations between samples and the solvent (DMSO). Initially titration experiments were performed by injecting Fe\textsuperscript{III} (Ent)\textsuperscript{3−} prepared at increasing concentrations of 3.12μM, 6.25μM, 12.5μM, 25μM, 50μM, 100μM in optimized running buffer at a flow rate of 30 μl/min. Following each injection, sensor chip surfaces were regenerated with a 30 second injection of 50% DMSO in running buffer and the kinetics were repeated for three times. Data were analyzed using Biacore T200 Evaluation software 2.0 version and 1:1 Binding Model (GE Healthcare). An affinity curve was generated manually by plotting the equilibrium binding response (R\textsubscript{eq}) against analyte concentration to obtain the dissociation constant (K\textsubscript{D}) values.

**Fluorescence emission measurements**

Room temperature fluorescence emission spectra were measured with a LS-55 Spectrofluorimeter (PerkinElmer corporate, USA) equipped with 1.0 cm quartz cells at room temperature with an excitation wavelength of 280 nm, and slit width of 5.0 nm for both excitation and emission. The concentration of OPH, and its variants OPH\textsuperscript{D301A}, OPH\textsuperscript{D301N} were fixed at 1 μM, and the concentrations of desferri-enterobactin and ferric-enterobactin dissolved in 0.1M phosphate buffer (pH 7.4) were varied from 1 to 11 μM. Three independent experiments were performed to obtain statistically significant data. The inner filter effect was corrected for absorption of exciting light and re-absorption of the emitted light. The binding constant was calculated using the maximum fluorescence intensity value at maximum emission wavelength (335nm) as described elsewhere (20).
Generation of OPH variants

Site-directed mutagenesis was performed to generate OPH variants by using a Q5 Site-Directed Mutagenesis Kit (Q5 SDM Kit, New England Bio Labs) following the manufacturer’s protocols. While generating OPH variants, the plasmid pUCOPH, was used as a template. The derivatives of pUCOPH encode mature form of OPH (OPH) without having any affinity tag. Similarly, plasmid pOPHV400, encoding preOPHCAviTag (precursor form of OPH) was used as a template while generating variants of OPH with signal peptide (precursor form of OPH). Complementary oligos having mutations at the desired position were designed and used for performing site-directed mutagenesis (Supplementary table 1). Plasmids pMD301A, pMD301N and pMK82A code for mature form of OPH variants OPHD301A, OPHD301N and OPHK82A respectively. Similarly, plasmids pPD301A, pPD301N and pPK82A code for precursor forms of OPH variants preOPH D301A, preOPH D301N and preOPH K82A. These plasmids were first transformed into E. coli strain, and the expression and stability of the OPH variants were assessed both by performing western blots and measuring OPH activity. The OPH variants were then purified (17) and used for performing further studies.

ExbD6xHis interactions with OPH variants

Pulldown assays were performed to assess interactions between OPH variants and ExbD6xHis following procedures optimized in our laboratory (3). Briefly the E. coli GS027 (pGS19) cells were transformed with the expression plasmids pMD301A, pMD301N and pMK82A and co-expression of OPH variants with ExbD6xHis were induced by adding 0.1mM IPTG. The clear lysate prepared from the induced cultures was taken to perform pull-down experiments and western blots were performed to detect OPH and ExbD6xHis (4).
Subcellular localization of OPH variants

While determining subcellular localization of wild type OPH and its variants, the *E. coli* (pGS19) cells were independently transformed with plasmids pOPHV400 (preOPH), pPD301A (preOPH$^{D301A}$), pPD301N (preOPH$^{D301N}$) or pPK82A (preOPH$^{K82A}$) coding precursor forms of OPH variants. The co-expression, subcellular fractionation and detection of ExbD$^{N6xHis}$ and OPH variants were performed following procedures described above (4).

Circular Dichroism (CD) Spectroscopy

CD spectra of OPH and OPH variants (OPH$^{D301A}$), (OPH$^{K82A}$) at a concentration of 100μg (0.0014 mM) were recorded with a Jasco J-810 spectropolarimeter (JAPAN) using a Quartz cuvette with path-length of 0.2 cm in a Nitrogen atmosphere at 25°C (21-23). Three scans were performed at a rate of 50nm/min and the spectral data were recorded in the range of 190 to 270nm.

Identification of ferric-enterobactin binding site.

i) Investigations on the catalytic site

We initially assumed ferric-enterobactin binds at the catalytic site of OPH and performed targeted docking of ferric-enterobactin using Autodock4.2 (24). The OPH structure was taken from its crystal structure (Pdb Id:1EYW) bound with the substrate analog triethyl phosphate. The 3D structure of ferric-enterobactin was retrieved from the crystal structure of a siderocalin-ferric-enterobactin complex (Pdb Id:3CMP). The active site of OPH (the receptor) was prepared for docking by removing all the water molecules except the one near the Zn metal ion at the catalytic site. The substrate analogue was also removed. The ferric-enterobactin structure was used as ligand during the docking. Thirty runs of docking were
attempted using the genetic algorithm in AutoDock. The docking poses were analyzed using AutoDock Tools. Pymol was used for visualization of the docking poses.

ii) Search for a potential ferric-enterobactin binding site

As the volume of the catalytic site was smaller than the volume of ferric-enterobactin, the catalytic site was not found to be suitable for docking. We have therefore searched for a potential ferric-enterobactin binding site in OPH. Since OPH exists as a homodimer (Pdb Id:1EZ2), we investigated the dimer structure for identification of potential ferric-enterobactin binding sites using the tools mentioned above.

Reconstitution of $s_f$TonBDT system in E. coli GS027

Initially we have deleted both exbD and tonB genes from Arctic Express strains following a P1 transduction method described elsewhere (25). The strain designated as E. coli GS027 was then tested for reconstitution of $s_f$TonBDT system. Functional TonB dependent transport (TonBDT) system contains an inner membrane associated Ton-complex and a transporter (TBDT) localized in the outer membrane (5). While reconstituting $s_f$TonBDT system E. coli GS027 cells were transformed with the expression plasmids pGS6 and pGS25 coding Ton-complex components $s_f$ExbB$^{\text{FLAG}}$, $s_f$ExbD$^{\text{CMyc}}$, $s_f$TonB$^{\text{C6xHis}}$ and outer membrane transporter $s_f$TBDT$^{\text{C6xHis}}$ respectively. The subcellular localization of these proteins was then established by performing western blots using tag-specific antibodies. The GS027 (pGS6 + pGS25) cells designated as GS029 were then used to independently transform with expression plasmids coding either wild type OPH (pOPHV400) or its variants preOPH$^{D301A}$ (pPD301A), preOPH$^{D301N}$ (pPD301N) and preOPH$^{K82A}$ (pPK82A). The resulting strains designated as GS030 (pGS6+pGS25+pOPHV400), GS031 (pGS6+pGS25+pPD301A) and GS032 (pGS6+pGS25+pPD301N) contain $s_f$TonBDT system either with wildtype OPH or with its variants preOPH$^{D301A}$, preOPH$^{D301N}$ and preOPH$^{K82A}$. 
Preparation of radiolabeled ferric-enterobactin

A stock solution of enterobactin (Sigma Aldrich, USA) was prepared by dissolving 1 mg of desferri-enterobactin in 100μl of DMSO. To prepare radiolabeled ferric-enterobactin, 3 μl of enterobactin stock solution was taken in a sterile eppendorf tube and 5 μl of ⁵⁵Fe was added from 0.2 μmol of ⁵⁵Fe stock (American Radiolabeled Chemicals, MO, USA; specific activity of 10.18 mCi / mg) and incubated at RT for 5 min. The contents were made up to 50 μl with 50mM HEPES buffer (pH 7.3) and the free ⁵⁵Fe was removed by passing the reaction mix through a Sephadex G-25 column. The radiolabeled ferric-enterobactin eluted in the flow through was collected and the radioactivity was determined by pipetting 2 μl into 5 ml of scintillation fluid [2,5-diphenyloxazole and 1, 4-bis (5-phenyl-2-oxazolyl) benzene] and measuring it in a Perkin Elmer Tri-Carb 2910TR scintillation counter.

Uptake of radiolabeled ferric-enterobactin

The E. coli GS027 and its derivatives GS029, GS030, GS031 and GS032 were grown to mid log phase in iron-sufficient medium and the expression of SfTonBDT components was induced for two hours following standard protocols. The induced cells were harvested and washed twice with the iron-free minimal salt medium. The cell pellet was then resuspended in an equal volume of iron limiting medium. Immediately, the culture flasks were shifted to 18°C and the expression of SfTonBDT components was induced for 12 hours by adding 1mM IPTG. After induction, the cells were harvested, washed extensively with iron free minimal medium and resuspended in the same culture medium to obtain a cell suspension with an OD₆₀₀nm of 1.0. Iron uptake was performed with this cell suspension. To an aliquote of cell suspension radiolabeled ferric-enterobactin (equivalent to 178 μmol of ⁵⁵Fe) was added incubated at 37°C for 2 h, with gentle shaking (150 rpm). The cells were harvested, extensively washed with 0.1M LiCl₂ followed by two washes with cold iron-free minimal media. After washing, the cell pellet was dried by keeping the pellet in a dry bath set at 60°C.
The dried cells were transferred into 5ml of scintillation fluid and the radioactivity determined in the LSS as described above. Wild-type Arctic Express cells treated in similar manner served as controls. Similar strategy was followed while measuring iron uptake in *S. wildii*, except that the cells were grown in a minimal medium that supported the growth of these cells (3, 14)

**RESULTS**

The study is designed to address the physiological role of OPH by taking into consideration its promiscuous lactonase activity and interactions with the inner membrane associated Ton-complex components ExbD and TonB (4). Ton-complex plays a role in the transport of nutrients, especially in the transport of ferric-enterobactin across the outer membrane. We hypothesized that the OPH played a role in the hydrolysis of the trilactone ring of enterobactin (Ent), a critical step required to release the iron from ferric-enterobactin. We experimentally validated this role by incubating OPH with pure ferric-enterobactin at different time intervals and analyzing the products by TLC to detect linear Ent formed due to lactonase activity of OPH. Instead of finding a discrete spot corresponding to linear version of Ent, we detected a streak that was reproducible and which was not observed in the lane spotted from a reaction mix prepared by omitting the OPH (Supplementary fig. S1). These results have prompted us to undertake further studies to detect existence of specific interactions between desferri-enterobactin / ferric-enterobactin and OPH.

**OPH shows stronger binding to ferric-enterobactin than to desferri-enterobactin:**

We performed three independent studies to ascertain interactions between OPH and desferri-enterobactin / ferric-enterobactin. Initially we performed both native PAGE (Fig. 1) and surface plasmon resonance (SPR) spectroscopy (Fig. 2, Panels A - D) to obtain qualitative and quantitative data on OPH interactions with both desferri-enterobactin and ferric-enterobactin (19). These results were then revalidated by performing fluorescence...
spectroscopy. Native PAGE has been successfully used to detect interactions between Ent and factor-H binding protein (fHbp) (19). The fHbp-Ent complex moved as an additional faster migrating band on native PAGE (19). While employing similar strategy we incubated pure OPH (10μM) with 100μM of ferric-enterobactin for 10 (Fig. 1, Panel B, lane 3) and 20 minutes (Fig. 1, Panel B, lane 4) respectively and analyzed the mixture on native PAGE. The corresponding immunoblots developed to detect OPH specific bands revealed the existence of a single OPH specific band in the lane loaded with pure OPH (Fig. 1, Panel B, lane 2), but in the reaction mixture containing both OPH and ferric-enterobactin there were two additional bands with greater mobility (Fig. 1, Panel B, lanes 3 & 4). These two additional OPH specific bands represent OPH-ferric-enterobactin complexes, likely to have formed due to interactions of ferric-enterobactin with OPH. One of them represents a complex formed between dimeric form of OPH and ferric-enterobactin and the second one appears to be a monomeric form of OPH, generated due to dissociation of dimeric OPH after interacting with ferric-enterobactin. Further, the intensity of the bands representing ferric-enterobactin-OPH complexes increased with the increase of incubation time (Fig. 1, Panel B, lanes 3 & 4). We have also performed similar studies to assess interactions of OPH with desferri-enterobactin. Surprisingly, intensity of the bands representing OPH-desferri-enterobactin complex was weak and the bands intensity failed to increase with the increase of incubation time, suggesting existence of a lesser affinity between OPH and desferri-enterobactin (Fig. 1, Panel A, lane 3 & 4). Based on these preliminary observations further studies were performed to gain better insights on OPH-ferric-enterobactin interactions. Initially, we obtained binding kinetics on OPH-ferric-enterobactin interactions by performing SPR analysis. Sensograms were generated by passing increased concentrations of either desferri-enterobactin (Fig. 2, Panel A) or ferric-enterobactin (Fig. 2, Panel B) over CM7 sensor chip (GE Healthcare) containing immobilized OPH. The values obtained from three independent experiments were
used to determine binding kinetics (Fig. 2, Panel C & D). Supporting the results of native PAGE, the binding kinetics have shown existence of a stronger affinity between OPH and ferric-enterobactin. The association, ($k_a = 246 \, (1/\text{Ms})$), and dissociation, ($k_d = 0.0073 \, (1/s)$) constant values obtained for ferric-enterobactin and OPH were significantly lower, when compared to the $k_a (249 \, (1/\text{Ms})$, $k_d (0.0302 \, (1/s)$ values obtained for OPH and desferri-enterobactin. Further, comparison of affinity constant ($K_D$) values obtained for ferric-enterobactin-OPH ($3.03 \, \text{E-05M} \pm 0.069 \, \text{E-05 M}$) and desferri-enterobactin-OPH ($9.10 \, \text{E-04 M} \pm 1.70 \, \text{E-04M}$) gave a clear indication on existence of lower affinity between OPH and desferri-enterobactin (Fig. 2, Panels C & D). In addition, a good correlation was also seen in the linear fitting curve drawn taking the difference of refractive index and concentrations of desferri-enterobactin (Fig. 2, Panel C) and ferric-enterobactin (Fig. 2, Panel D).

Additionally, we have also measured intrinsic fluorescence emission maximum intensity for OPH in presence of different concentrations of desferri-enterobactin and ferric-enterobactin. Basically, the desferri-enterobactin or ferric-enterobactin show no fluorescence (Fig. 2, Panels E & F). However, when they were added to OPH in increasing concentrations (1-11 μM) maximum fluorescence emission obtained at 335nm was proportionately decreased (Fig. 2, Panels E & F). This observation clearly showed concentration dependent quenching of fluorescence. Such decrease in fluorescence intensity is due to interaction of excited state desferri-enterobactin or ferric-enterobactin (fluorophores) with its surrounding OPH molecules. Supporting this proposition, a total quench in the fluorescence of OPH was observed at maximum concentrations of desferri-enterobactin and ferric-enterobactin (Fig. 2, Panels E & F). Further, a good linear relationship was seen between concentration of desferri-enterobactin and ferric-enterobactin and fluorescence quenching of OPH (Fig. 2, Panels E & F). The binding dissociation constants as calculated from the Y-intercept of the slope obtained with OPH at different concentrations of desferri-enterobactin was $2.00803 \, \text{E-06} \pm$
3.51188E-11 M. Likewise, the binding dissociation constants for ferric-enterobactin with OPH found to be 4.31034E-07 ±5.7449E-11 M (Fig. 2, Panels E & F). The association/binding constants calculated for OPH indicated existence of only one binding site for interacting with Ent/ ferric-enterobactin. Interestingly, the kinetic values determined by using fluorescence emission showed very good correlation with binding kinetics determined by SPR data (Fig. 2, Panel C & D). All three independent studies involving BN-PAGE, SPR and fluorescence emission indicated existence of interactions between OPH and Ent and the interactions found between ferric-enterobactin and OPH appears to be much stronger than desferri-enterobactin.

**Active site of OPH has no role in Ent interactions**

Initially we assumed involvement of OPH active site in establishing interactions with Ent. We therefore generated variants of OPH with altered active site by substituting active site aspartate (D301) with amino acids having either light or bulky side chains. These OPH variants, OPH$_{D301A}$ and OPH$_{D301N}$ retained stability, secondary structure like native OPH (Fig. 3, Panels A & C) and failed to show OPH activity (Fig. 3, Panel B, lanes 2 & 3). We took these triesterase negative OPH variants, OPH$_{D301A}$, OPH$_{D301N}$ to test their ferric-enterobactin binding ability by measuring both SPR and fluorescence emission. Interestingly these two independent studies have shown the existence of ferric-enterobactin binding ability both for OPH$_{D301A}$ and OPH$_{D301N}$ (Fig. 4, Panels A to F). The OPH$_{D301A}$ showed better ferric-enterobactin binding efficiency than OPH$_{D301N}$ and its binding efficiency was comparable to the wild type OPH (Fig. 4, Panels A, C & E). The existing data fails to explain reasons to have such a weak affinity between OPH$_{D301N}$ and ferric-enterobactin (Fig. 4, Panels B, D & F), but prove beyond reasonable doubt the existence of high affinity interactions between ferric-enterobactin and triesterase negative OPH variants, probably through a site independent of OPH active site.
OPH contains A novel ferric-enterobactin binding site

Since active site mutations failed to influence OPH-Ent interactions, we presumed that there exists an exclusive site in OPH for binding with ferric-enterobactin. In order to investigate this possibility, we performed blind docking of ferric-enterobactin on OPH using AutoDock. We obtained seven different potential ferric-enterobactin binding site possibilities in OPH, of which the PARS server supported only three of them (26). Among the three sites, two were identical to each other, situated on both the monomers related by the dimer symmetry (Fig. 5), with the third seen at the dimer interface. The sites on the two monomers corresponded to the best binding energy (Fig. 5) and these sites were regarded as potential binding sites for ferric-enterobactin that were used for all further studies as mentioned below.

To get a better model of a potential OPH-ferric-enterobactin complex with regard to the binding site as mentioned above we did targeted docking at this site as described in Methods. The best pose obtained from the docking study is stabilized by hydrogen bonding interactions by side chains of Lys82 and Arg85 placed between two of the three catechol rings as well as the hydrophobic contacts made by Asp315, Met314, Phe304, Asp318, Arg319, Arg89 and Pro322 as shown in Fig. 5, Panels B & C. Such interactions have been reported in Neisseria meningitides (19) demonstrating the interaction of xenosiderophores with Factor H binding protein (fHbp). These in silico predictions were validated by generating OPH variant with substitutions at the lysine residue believed to be critical for binding to ferric-enterobactin. The OPH variant OPH\textsuperscript{K82A}, encoded by pMK82A, retained OPH activity (Fig. 6, Panel A-II, Lane 2), stability (Fig. 6, Panel A-I, Lane 3) and other structural properties (Fig. 6, Panel B) at a level comparable to the wild type enzyme (Fig. 6, Panel A-I, lane 2; A-II, lane 1 & Panel B). However, the OPH\textsuperscript{K82A} failed to interact with ferric-enterobactin. There was neither a band that corresponded to the OPH\textsuperscript{K82A}-ferric-
enterobactin complex on native PAGE (Fig. 6, Panel C) nor was there a refraction in SPR when Ent was passed over OPH$^{K82A}$ suggesting its inability to interact with ferric-enterobactin (Fig. 6, Panel D). The experimental data clearly supported in silico predictions which suggested the existence of an exclusive ferric-enterobactin binding site in OPH (Fig. 5, A-C).

The $S_f$TonBDT system complements E. coli GS027.

After gaining consistent positive results on OPH and ferric-enterobactin interactions, we conducted further experiments to gather evidence on the role of OPH in outer membrane transport, specifically in the transport of ferric-enterobactin. Initially, we have created mutant strain of E. coli Arctic Express (GS027) defective in TonB dependent transport (TonBDT) system by deleting $exbD$ and $tonB$ genes. This strain, GS027 was then complemented with $S_f$Ton-complex components $S_f$ExbB$^{NFLAG}$, $S_f$ExbD$^{CMyc}$ and $S_f$TonB$^{C6His}$ by transforming expression plasmid pGS6. Before proceeding with further experiments, the subcellular localization of $S_f$Ton-complex components in strain GS028 (GS027 transformed with pGS6) was ascertained by performing western blots using epitope specific antibodies. The western blot results have clearly indicated presence of $S_f$TonB$^{C6His}$, $S_f$ExbD$^{CMyc}$ and $S_f$ExbB$^{NFLAG}$ in the membrane fraction obtained from strain GS028 (Fig. 7, Panel A-II, III & IV, Lane M). The GS028 cells were then used to co-express outer membrane transporter (TBDT) co-purified with OPH (4), by transforming $S_f$TBDT$^{C6His}$ coding pGS25 into GS028 cells. The resulting GS029 (pGS6+pGS25) strain codes both inner membrane associated Ton-complex and outer membrane transporter, $S_f$TBDT$^{C6His}$. Interestingly, all of them have successfully targeted the membrane in induced cultures of GS029 (Fig. 7, Panel A-II, III, IV & V, lane M) and complemented the strains growth defect in iron deficient minimal medium suggesting restoration of a functional TonBDT system in E. coli $exbB$ and $tonB$ negative mutants (Fig. 7, Panel B). Finally, the GS029 (pGS6+pGS25) cells were used to reconstitute $S_f$TonBDT.
system with OPH by transforming the strain with pOPHV400. The ectopically expressed precursor form of OPH remains in cytoplasm as an unprocessed protein, if it is not interacted with Ton-components to form a four component Ton-complex (4). Since, the GS030 cells contain necessary Ton-complex components, the plasmid, pOPHV400 coded OPHCAviTag targeted the membrane indicating successful reconstitution of OPH containing SfTonBDT system in E. coli GS030 cells (Fig. 7, Panel VI, lane M). Since TonBDT system is known to transport ferric-enterobactin, the three strains, GS027, GS029 and GS030, together with the wild type, were grown under iron limiting conditions and growth was monitored. Both GS029 (pGS6+pGS25) and GS030 (pGS6+pGS25+pOPHV400) grew successfully under iron-limiting conditions. However, the strain GS030 expressing SfTonBDT system with OPHCAviTag showed better growth than the strain GS029 having without OPHCAviTag (Fig. 7, Panel B).

**OPH-dependent increase in iron uptake**

In order to test if the increased growth in these cells was due to increased iron uptake, we incubated cells acclimatized to the low iron-containing medium for two hours with pure radiolabeled ferric-enterobactin. As expected, no $^{55}\text{Fe}$ was found in ΔexbD, ΔtonB (GS027) cells (Fig. 7, Panel C, lane 2). Nearly 28,000 pico moles of iron was found in wild-type cells (Fig. 7, Panel C, lane 1), whilst cells expressing SfTonBDT system without OPH showed 26,000 pico moles of iron uptake (Fig. 7, Panel C, lane 3) and cells having SfTonBDT system with OPH showed a two-fold increase in iron uptake to 48,000 pico moles (Fig. 7, Panel C, lane 4). Hence the presence of OPH enhanced TonBDT-dependent iron uptake.

**Both triesterase and Ent binding activities are critical for increased iron uptake**

A clear positive influence of OPH was seen on growth and iron uptake of E. coli GS030 (pGS6+pGS25+pOPHV400) cells having SfTonBDT system with OPH. However, it was not
clear if the observed enhancement in growth and iron uptake was due to triesterase/ ferric-enterobactin binding activity of OPH. We have therefore reconstituted $\gamma$TonBDT system in *E. coli* GS029 cells with OPH variants that failed to show triesterase activity (OPH$^{D301A}$ and OPH$^{D301N}$) and ferric-enterobactin binding ability (OPH$^{K82A}$). The OPH and ExbD interactions are critical for membrane targeting of OPH in *E. coli* (4). Before proceeding to reconstitute $\gamma$TonBDT system in GS029 cells with OPH variants, we tested if they have retained the ability to interact with ExbD. Pulldown assays were performed using the cell lysates obtained from GS027 cells co-expressing OPH variants and ExbD$^{N6xHis}$. Surprisingly, the OPH$^{K82A}$ which lost ferric-enterobactin binding ability failed to interact with ExbD (Fig. 8, Panel C, row-II lane 12). In the absence of these interactions, most of the preOPHK82A co-expressed in GS027 cells along with ExbD$^{N6xHis}$ remained in cytoplasm, indicating the significance of ferric-enterobactin binding site in establishing functional TonB complex required for iron uptake (Fig. 9, Panel D lane M). In contrast, both OPH$^{D301A}$ and OPH$^{D301N}$ have retained ability to cross-talk with ExbD$^{N6xHis}$ (Fig. 8, panels A & B, row-II, lane 12). When cell lysates prepared from GS027 (pGS19+pMD301A) co-expressing both OPH$^{D301A}$ and ExbD$^{N6xHis}$ was passed through Ni-NTA magnetic beads OPH$^{D301A}$ got copurified with ExbD$^{N6xHis}$ (Fig. 8, Panel A-II lane 12). Similarly, OPH$^{D301N}$ got copurified when lysate containing OPH$^{D301N}$ and ExbD$^{N6xHis}$ was passed through the metal ion affinity column (Fig. 8, Panel B-II lane 12), suggesting existence of interactions between OPH variants, OPH$^{D301A}$, OPH$^{D301N}$ and ExbD$^{N6xHis}$ (Fig. 8, Panel A & B, rows I & II). In presence of these interactions the preOPHD301A and preOPHD301N have successfully targeted to the inner membrane in *E. coli* GS027 in $\gamma$ExbD positive background (Fig. 9, Panel B, C, lane M). Therefore, reconstitution of $\gamma$TonBDT system was possible with OPH$^{D301A}$, OPH$^{D301N}$ in *E. coli*. The GS031 cells having with either (pGS6+pGS25+ pPD301A) or GS032 cells (pGS6+pGS25+ pPD301N) were then grown under iron limiting conditions and used for studying iron uptake.
Surprisingly the OPH dependent enhanced iron uptake was not seen in cells having s/TonBDT system reconstituted with OPH$_{D301A}$ and OPH$_{D301N}$ suggesting that 301 aspartate that contributes for triesterase activity is critical for OPH dependent enhanced iron uptake in *E. coli* GS031 and GS032 cells (Fig. 7, Panel C lanes 5, 6).

Since OPH dependent increase in iron dependent growth was seen in *E. coli* cells reconstituted with s/TonBDT system we wished to find out if OPH has similar influence on the iron uptake in *S. fuliginis* cells. We have made several unsuccessful attempts to generate opd negative mutant of *S. fuliginis* following standard procedures (2). However, we were able to obtain opd negative mutant of *Sphingopyxis wildii* following similar strategies (2). Identical opd genes exist both in *S. fuliginis* and *S. wildii* (9). We therefore used opd negative strains of *S. wildii* to test OPH role in ferric-enterobactin dependent iron uptake. Initially we acclimatized opd negative and positive cells of *S. wildii* to iron limiting conditions by growing them in a medium containing low iron (0.02 μg Fe / mL / 0.36 μM) and monitored their growth. The opd mutant of *S. wildii* showed retarded growth when compared to the wild type cells, the kind of growth behaviour typically seen in GS029 cells having s/TonBDT system without OPH. After studying the growth behaviour equal number of cells were taken from these cultures and incubated with radiolabelled ferric-enterobactin to see accumulation of $^{55}$Fe in opd positive and opd negative background. Interestingly, the wild type cells took up 31586 picomoles of iron as against 11497 picomoles found in opd negative mutants (Fig. 10, Panel B). Although mechanistic details are elusive, results generated in this study clearly suggest a role for OPH in ferric-enterobactin mediated iron uptake in *S. fuliginis* ATCC 27551. Due to lateral mobility of OPH coding opd gene identical OPH coding genes were present in soil bacteria that share very weak taxonomic relationship (27, 28). It is not known if OPH plays a similar role in these bacteria. Further studies are required to gain better insights on the role of OPH in outer membrane transport of nutrients.
DISCUSSION

Transport of nutrients through energy deprived outer membrane of Gram negative bacteria acquired lot of significance. It plays a critical role in transport of scarcely available nutrients such as vitamin B12, siderophores, heme, host iron-binding proteins, maltodextrins, nickel etc in the environment (5, 29-31). The outer membrane transport is facilitated by an inner membrane localized Ton-complex (TonB, ExbB/ExbD) and an outer membrane transporter, called TonB-dependent transporter (TBDT). The unique structural features of TBDT facilitate transport of a variety of nutrients. TBDT contains a membrane-spanning barrel domain comprising 22 anti-parallel \( \beta \)-strands, which is bigger than the barrel domain found in porins (32). The N-terminus of TBDT contains a plug domain that seals the barrel and prevents passage of solutes into the periplasmic space. Substrate binding induces conformational change and exposes the TonB box of TBDT to facilitate interaction between TBDT and the inner-membrane-associated TonB. The energy derived from the inner-membrane-associated Ton complex, comprising TonB, ExbB and ExbD, is transduced to TBDT through TonB (32). The exact mechanism by which TonB transduces energy to TBDT is still not known, although two models have been proposed. One of them is a shuttle model in which energized TonB exits the inner membrane and shuttles across the periplasm to transduce energy to TBDT (30, 33, 34). The second one is a pulling model which proposes that TonB remains associated to the inner membrane and spans the periplasmic space to connect with the TonB box of TBDT. The conformational changes of energized TonB then pull the plug domain of TBDT into the periplasmic space and the substrate bound to TBDT translocates to the periplasmic space (5).

Unlike cytoplasmic membrane (CM) transporters and transcription factors there is no correlation between genome size and the number of TBDT species in an organism (35), rather the number of TBDTs is dependent on an organism’s physiology and ecological niche.
Pathogenic *E. coli* strains encode twice the number of TBDTs found in non-pathogenic *E. coli* strains, and a greater number of TBDTs appears to contribute to efficient scavenging of a variety of nutrients either in densely populated microbial niches or in nutrient-poor environments (36). In the genome of *S. fuliginis* 87 TBDT coding sequences have been predicted with just one copy of ExbB, 3 copies of ExbD and four copies of TonB coding sequences. This increase in TBDTs seems to be part of the organism’s survival strategy in a nutrient-diverse soil environment and indicates the existence of efficient and robust mechanisms for transport of rare carbon sources and scarcely available nutrients.

Despite the observed structural diversity in TBDTs, the energy harnessing and transducing Ton complex of TonBDT is highly conserved. In Gram-negative bacteria it is a ternary complex comprising TonB, ExbB and ExbD in a 1:7:2 ratio (32). As opposed to this established notion, in *S. fuliginis* we find a four-component TonB complex comprising TonB, ExbB, ExbD and OPH (4). The existence of this quaternary Ton complex appears to be advantageous to the cell as it contributes to better cell growth and more efficient iron uptake (Fig. 7, Panels B & C). However, the mechanistic details of how OPH contributes to an increased iron uptake are not known.

In the TonBDT systems known to date, the transport of ferric-enterobactin and subsequent release of ferric ions bound to Ent are independent events. In certain cases, the trilactone ring of ferric-enterobactin transported to periplasmic space / cytoplasm is hydrolysed to facilitate the release of ferric iron (37). Although this mechanism is found in certain Gram negative bacteria it is considered to be less efficient to the cell (38). The Ent made in the cell cannot be recycled and the cell has to make a fresh Ent for transport of every single iron atom. Protonation induces huge change in coordination mode of ferric-enterobactin besides altering its molecular shape (38). Such molecular events weaken association of ferric ions with Ent facilitating their release at physiological pH (39). Release
of iron from ferric-enterobactin due to protonation is physiologically advantageous to the cell as it promotes recycling of Ent (38).

OPH interacts with ferric-enterobactin (Fig. 2, Panel B) and it also contributes for increased iron uptake in *E. coli* GS030 (*s/TonBDT* system + OPH) cells (Fig. 7, Panel C). However, the precise mechanism by which OPH contributes for the enhanced iron uptake in *E. coli* GS030 (*s/TonBDT* system + OPH) cells is unclear. Transport of ferric-enterobactin in *E. coli* GS029 is independent of OPH (Fig. 7, Panel C) and merely accelerates the uptake process. The reasons for enhanced iron uptake in presence of OPH might be due to release of the ferric ion ferric-enterobactin. As Ent is not a substrate for OPH, the OPH dependent increase might be due to protonation of ferric-enterobactin complex by involving its binuclear metal centre in reactions requiring electron or proton transfer. Our previous studies have established the interaction of OPH with both the energy-harnessing (ExbD) and energy-transducing (TonB), components of *s/TonBDT* (4). The existence of a binuclear metal centre in association with a proton channel (ExbB/ExbD) and an energy-transducing (TonB) component appears to have functional significance. It suggests a role for OPH in redox reactions, which might directly contribute to efficient harnessing and transduction of energy to the outer membrane transporter, TBDT. This proposition gains acceptance as the GS031, GS032 cells consisting *s/TonBDT* system with having preOPH<sup>D301A</sup> or preOPH<sup>D301N</sup> failed to show enhanced iron uptake (Fig. 7, Panel C). The aspartate found at 301 position (D301) is part of OPH active site that contributes for the triesterase activity (Fig. 3, Panel B). It coordinates with the binuclear metal centre of OPH. Substitution of D301 with alanine or asparagine affected both triesterase activity and iron uptake in GS031 and GS032 (*s/TonBDT* system with preOPH<sup>D301A</sup>, *s/TonBDT* system with preOPH<sup>D301N</sup>) cells. The amount of iron taken up by GS031 and GS032 (*s/TonBDT* system with preOPH<sup>D301A</sup>, *s/TonBDT* system with preOPH<sup>D301N</sup>) cells is equal to the amount of iron transported into GS029 cells having
TonBDT system reconstituted without OPH. If these results are seen together with the protonation dependent weakening of ferric-enterobactin interactions, the proposed mechanism gains strength. However, further studies are required to gain experimental evidence for the proposed mechanism.

**AUTHOR CONTRIBUTIONS:** DS conceived the idea and designed experiments. HP, RG, GVK and ERD performed experiments. HAN supervised bioinformatic studies. MS and RS guided to perform iron uptake studies and fluorescence emission microscopy respectively. DS wrote the manuscript.

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Table 1: Strains and plasmids used in the study

| Strains               | Genotype or Phenotype                                      | Reference or Source |
|-----------------------|-----------------------------------------------------------|---------------------|
| E. coli DH5α          | λsupE44 ΔlacU169 (Δ80 lacZΔM15)                           | (40)                |
| Sphingopyxis wildii   | Sm′, PmB′, opd′                                           | (41)                |
| Sphingopyxis wildii DSO10 | Sm′, Te′, PmB′, opd′ (opd::tet)                             | (2)                |
| Arctic Express        | E. coli B FompThsdS (rB mB) dcm_Tel gal endA Hte (cpn10 cpn60 Gent) | Agilent Technologies |
| E. coli K-12 MG1655   | Wild type strain, F−, λ−, rph−                            | (42)                |
| E. coli GS023         | Gm′, Km′. Arctic Express, exbD::km                         | This study          |
| E. coli GS024         | Gm′, ΔexbD. Generated by deleting Km resistant cassette from exbD::Km | This study          |
| E. coli GS026         | Gm′, Km′. Generated by inserting Km cassette into tonB. ΔexbD, tonB::Km | This study          |
| E. coli GS027         | Gm′. Generated by deleting Km cassette from tonB::Km of E. coli GS026. ΔexbD, ΔtonB. | This study          |
| E. coli GS028         | Gm′, Amp′ Km′. Cells having reconstituted sTonBDT system (pGS6) | This study          |
| E. coli GS029         | Gm′, Amp′ Km′. Cells having reconstituted sTonBDT system (pGS6+pGS25) | This study          |
| E. coli GS030         | Gm′, Amp′ Km′. Cm′. Cells having reconstituted sTonBDT system with OPH (pGS6+pGS25+pOPH400) | This study          |
| E. coli GS031         | Gm′, Amp′ Km′. Cm′. Cells having reconstituted sTonBDT system with OPHD301A (pGS6+pGS25+pPD301A) | This study          |
| E. coli GS032         | Gm′, Amp′ Km′. Cm′. Cells having reconstituted sTonBDT system with OPHD301N (pGS6+pGS25+pPD301N) | This study          |
| Plasmid Name | Genotype or phenotype                                                                                                                                                                                                 | Reference or Source |
|-------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
| pMMB206     | Cm\(^{r}\), broad host range, low copy number, expression vector.                                                                                                                                                     | (43)                |
| pETDuet1    | Amp\(^{r}\), The T7 promoter driven expression vector. Contains two multiple cloning sites. Facilitates co-expression of two proteins                                                                                     | Novagen             |
| pCP20       | Amp\(^{r}\), Cm\(^{r}\), low copy number plasmid, FLP\(^{r}\), P\(\lambda\) ci857\(^{r}\), P\(\lambda\) PR Rep\(^{r}\),                                                                                             | (25)                |
| pACYC177    | Km\(^{r}\), low copy number, broad host range vector                                                                                                                                                                  | (44)                |
| pGS2N       | Km\(^{r}\), low copy number, broad host range expression vector, generated by ligating MCS of pRSETA to a fragment containing kanamycin resistant gene and oriV of pACYC177. When cloned it codes for a protein with N-terminal 6His-tag | (4)                 |
| pSM5        | Cm\(^{r}\), Expression plasmid. Codes preOPH\(^{r}\) 6His. Generated by cloning opd gene amplified from pPHYS400 in pMMB206 as EcoRI and HindIII fragment.                                                             | (45)                |
| pUCOPH      | Amp\(^{r}\), opd cloned in pUC19 vector under the control of constitutive promoter.                                                                                                                                 18                   |
| pPHV400     | Cm\(^{r}\), AviTag coding sequence inserted as XhoI and HindIII fragment inframe of opd gene of pSM5, codes for OPH\(^{r}\) AviTag                                                                                      |                    |
| pGS6        | Amp\(^{r}\), Expression plasmid. Generated by cloning exbBD operon in pGS5 as EcoRI and SacI fragment. The exbBD operon is taken from pGS23. Codes for TonB\(^{r}\) 6His\(^{r}\) and ExbB\(^{r}\) ExbD\(^{r}\) 6His\(^{r}\) | (3)                 |
| pGS19       | Km\(^{r}\), Expression plasmid. Generated by cloning exbD gene amplified from Sphingobium fuliginis ATCC 27551 by using RG4 FP and RG4 RP as forward and reverse primers in pGS2N. Codes for ExbD\(^{r}\) 6His\(^{r}\) | (4)                 |
| pGS2C       | Km\(^{r}\), low copy number, broad host range expression vector, generated by ligating MCS of pET28a and kanamycin resistant gene and oriV of pACYC177. When cloned it codes for a protein with C-terminal 6xHis-tag. | This Study          |
| pGS25       | Km\(^{r}\), The T7 promoter driven expression plasmid. Generated by cloning tbdt in pGS2C. The tbdt was amplified from Sphingobium fuliginis ATCC 27551 by using RG13 FP and RG13 RP as forward and reverse primers. Codes for TBTD\(^{r}\) ExbD\(^{r}\) 6His\(^{r}\) | This Study          |
| pMD301A     | Amp\(^{r}\), Expression plasmid. The pUCOPH derivative. Codes for OPH\(^{D301A}\)                                                                                                                                     | This Study          |
| pMD301N     | Amp\(^{r}\), Expression plasmid. The pUCOPH derivative. Codes for OPH\(^{D301N}\)                                                                                                                                     | This Study          |
| pMK82A      | Amp\(^{r}\), Expression plasmid. The pUCOPH derivative. Codes for OPH\(^{K82A}\)                                                                                                                                      | This Study          |
| pPD301A     | Cm\(^{r}\), Expression plasmid. The pPHV400 derivative. Codes for preOPH\(^{D301A}\)                                                                                                                                    | This Study          |
| pPD301N     | Cm\(^{r}\), Expression plasmid. The pPHV400 derivative. Codes for preOPH\(^{D301N}\)                                                                                                                                     | This Study          |
| pPK82A      | Cm\(^{r}\), Expression plasmid. The pPHV400 derivative. Codes for preOPH\(^{K82A}\)                                                                                                                                     | This Study          |
Figure 1: Demonstration of OPH: ferri-enterobactin interaction by native PAGE.
Purified OPH (10 μM) was added to separate reaction mixtures containing 100 μM of desferri-enterobactin and ferric-enterobactin respectively. They were incubated for varying time periods, separated by native PAGE on a 4-16% Bis-Tris gradient gel and then subjected to immunoblotting with anti-OPH antibodies. Panels A & B are the immunoblots representing OPH: desferri-enterobactin and OPH: ferric-enterobactin interactions respectively, with lanes 3 and 4 in each panel representing the products observed after 10 and 20 minutes of incubation; lane 2 was loaded with OPH alone and lane 1 indicates mobility pattern of the native gel protein molecular markers obtained from a stained gel. Faster migrating bands in lanes 3 and 4 indicate monomeric (*) and dimeric (**) forms of OPH complexed with Fe-Ent.

Figure 2: SPR and spectrofluorimetric analysis of OPH: ferri-enterobactin interactions.
Purified OPH was immobilized on the CM-7 chip and two identical experiments performed with increasing concentrations of desferri- (Panel A) and ferric-enterobactin (Panel B) respectively. Panels C & D represent the kinetic model based on a mathematical algorithm of the sensorgrams that was generated with the BiacoreT200 evaluation software. This was used to derive the dissociation constant K_D. The emission spectra derived from spectrofluorimetric analysis is represented in Panels E & F and the quenching seen upon increasing the concentration of the siderophore (1 to 11 μM) can be seen. The inset shows the modified Stern–Volmer plots obtained by plotting log [Q] vs log [dF/F] values, with the slope giving the number of binding sites. Using the logK values from the intercept on the Y axis, the binding constant values were calculated. The arrow indicates the negligible fluorescence emission of buffer / desferri- / ferric-enterobactin.

Figure 3: Characterisation of the recombinant OPH and its variants.
Panel A shows the homogeneity of the recombinant OPH (lane 2) and the two variants OPH<sup>D301A</sup> (lane 3), OPH<sup>D301N</sup> (lane 4) as analysed by SDS-PAGE on a 12.5% gel; lane 1 represents the molecular marker. Panel B represents the conventional enzymatic assay of OPH and its two variants performed using methyl parathion as the substrate. Panel C indicates the superimposed CD spectra recorded for OPH and its variants.
Figure 4: SPR and spectrofluorimetric assays for assaying interactions of OPH variants with ferric-enterobactin.

The experiments were performed essentially as detailed in Figure 2 for the wild type OPH, with Panels A and B representing the sensograms of OPH$^{D301A}$ and OPH$^{D301N}$ respectively and Panels C & D the derivation of the K$_D$ values from the graphs generated from the ‘response units’ vs the ferric-enterobactin concentration. Spectrofluorimetric assay, performed as for the wild type OPH is represented in Panels E and F for OPH$^{D301A}$ and OPH$^{D301N}$ respectively, with the determination of the binding sites from the Stern–Volmer plots (shown as insets) generated as described in Figure 2. Arrow indicates baseline emission of the ferric-enterobactin.

Fig. 5: Ferric-enterobactin binding site in OPH: in silico analysis

Panel A shows the molecular surface of the dimer crystal structure of OPH (PDB Id:1EZ2) with the best predicted ferric-enterobactin binding site. As mentioned in the text this ferric-enterobactin binding site is found on each monomer, therefore, the dimer structure harbours two identical binding sites, each related to the other by dimer symmetry. For the sake of clarity only one binding site is shown. The two monomers are shown in two different colors viz., yellow and grey colors and for the purpose of clarity the ferric-enterobactin binding site is shown in magenta colour. The ferric-enterobactin is shown in ball and stick representation.

Panel B shows the predicted ferric-enterobactin binding site after zooming in. The ligand ferric-enterobactin and the binding site are shown in ball and stick and ribbon representations respectively along with the ligand-OPH interactions.

Panel C shows the interacting residues of OPH with ferric-enterobactin at the predicted binding site. Lys82 and Arg85 placed between two of the three catechol rings make hydrogen bond interactions with ferric-enterobactin and the other residues (Asp315, Met314, Phe304, Asp318, Arg319, Arg89 and Pro322) make hydrophobic contacts.

Panels A and B were generated using PyMol 2.0 (Schrodinger, LLC ) and Panel C was generated using LigPlot+ tool (46).
Figure 6: Experimental validation of the catalytic binding site for ferric-enterobactin in OPH.

The newly generated variant OPH\textsuperscript{K82A} and the wild type OPH were first checked for homogeneity (Panel A-I, enzymatic activity using methyl parathion as substrate (Panel A-II) and changes in secondary structure by CD spectral analysis (Panel B). The interactions between OPH\textsuperscript{K82A} on ferric-enterobactin were determined following procedures detailed in methods section. The reaction mixtures containing OPH\textsuperscript{K82A} and ferric-enterobactin incubated for 10 (lane 2) and 20 minutes (lane 3) were subjected to native PAGE and western blot analysis, as described in Figure 1. Panel C represents the corresponding immunoblot showing the presence of OPH\textsuperscript{K82A} specific bands. The poor affinity of the variant OPH\textsuperscript{K82A} for ferric-enterobactin was confirmed by SPR analysis, with Panel D showing negligible binding even at higher concentrations of the siderophore.

Figure 7: Generation of the recombinant \textit{E. coli} GS030 and demonstration of OPH influence on uptake of radiolabeled ferric-enterobactin.

Recombinant \textit{E. coli} GS030 was generated by reconstitution of the \textit{s}fr\textit{TonBDT} system in \textit{E. coli} GS027 and here Panel A shows the presence of the various components of the TonBDT systems by developing immunoblots using epitope-specific antibodies. Panel A-I shows the Coomassie Blue stained proteins of whole-cell lysate (WL), cytoplasm (C) and membrane (M) fractions of this recombinant strain. The immunoblots were developed with the respective antibodies to assess the expression and subcellular localization of \textit{s}fr\textit{TonBC6His} (II), \textit{s}fr\textit{ExbDCMyc} (III), \textit{s}fr\textit{ExbBNFLAG} (IV), \textit{s}fr\textit{TBDTC6His} (V), and OPH\textsuperscript{CAviTag} (VI) respectively. Panel B shows the growth of \textit{exbB}, \textit{tonB} negative GS027 cells (▲) complemented with \textit{s}fr\textit{TonBDT} system without (■) and with OPH (○) in iron limiting minimal medium. The strain GS027 (pGS6+pGS25), designated as GS029 is complemented with \textit{s}fr\textit{TonBDT} system without OPH, whereas the strain GS027 (pGS6+pGS25+pOPHV400), designated as GS030 contains \textit{s}fr\textit{TonBDT} with OPH. Panel C shows iron uptake in wild type \textit{E. coli} Arctic Express cells (1) and its GS027 derivative generated by deleting \textit{exbB} and \textit{tonB} genes (2). Lanes 3 and 4 show iron uptake in GS027 cells complemented with \textit{s}fr\textit{TonBDT} system without OPH (GS029) and with OPH (GS030), respectively. Lanes 5 and 6 indicate iron uptake in cells having a reconstituted \textit{s}fr\textit{TonBDT} system with OPH variants OPH\textsuperscript{D301A} (GS031) and OPH\textsuperscript{D301N} (GS032), respectively.
Fig. 8: Interactions with OPH variants and ExbD.

Four *E. coli* strains were generated by transforming GS027 (pGS19) cells with plasmids pUCOPH, pMD301A, pMD301N and pMK82A. Co-expression of ExbD\textsuperscript{N6xHis} + OPH (panels A to C), ExbD\textsuperscript{N6xHis} + OPH\textsuperscript{D301A} (Panel A), ExbD\textsuperscript{N6xHis} + OPH\textsuperscript{D301N} (Panel B) and ExbD\textsuperscript{N6xHis} + OPH\textsuperscript{K82A} (Panel C) was induced in these cells by adding 1mM IPTG. Panels A to C indicate pulldown assays performed using lysates (CL\textsuperscript{C}) of GS027 cells co-expressing ExbD\textsuperscript{N6xHis} + OPH (positive control), ExbD\textsuperscript{N6xHis} + OPH\textsuperscript{D301A} (Panel A), ExbD\textsuperscript{N6xHis} + OPH\textsuperscript{D301N} (Panel B) and ExbD\textsuperscript{N6xHis} + OPH\textsuperscript{K82A} (Panel C). The cell lysates (CL\textsuperscript{C}) were passed through Ni-NTA beads, and the Flow through (FT), wash (W) and elution (E\textsuperscript{C}) fractions were collected and analyzed on SDS-PAGE (12.5%). Rows I and II represent corresponding western blots performed using either anti-His antibodies (row-I) or anti-OPH antibodies (row-II) to detect ExbD\textsuperscript{N6xHis} and OPH or its variants. Control cell lysates containing either only OPH or its variants (CL\textsuperscript{O}) or ExbD\textsuperscript{N6xHis} (CL\textsuperscript{E}) treated similarly, and the elution fractions collected from the Ni-NTA beads were loaded in lanes designated as E\textsuperscript{O} and E\textsuperscript{E} respectively. Lanes 1 to 8 represent pulldown assays of control lysates containing wild type OPH + ExbD\textsuperscript{N6xHis}. Lanes 9 to 16 represent test pulldown assays performed using lysates containing ExbD\textsuperscript{N6xHis} + OPH\textsuperscript{D301A} (Panel A), ExbD\textsuperscript{N6xHis} + OPH\textsuperscript{D301N} (Panel B) ExbD\textsuperscript{N6xHis} + OPH\textsuperscript{K82A} (Panel C). The rectangle box in panel C shows the inability of OPH\textsuperscript{K82A} to form a stable complex with ExbD\textsuperscript{N6xHis}. 
Figure 9: SDS-PAGE and Western blot analysis to demonstrate the membrane targeting of OPH and its variants.

These strains described in figure 8 co-expressing ExbD<sup>N6xHis</sup> + preOPH (Panel A), ExbD<sup>N6xHis</sup> + preOPH<sup>D301A</sup> (Panel B), ExbD<sup>N6xHis</sup> + preOPH<sup>D301N</sup> (Panel C) and ExbD<sup>N6xHis</sup> + preOPH<sup>K82A</sup> (Panel D) were fractionated as described in methods. The whole cell lysate (WL), cytoplasmic (C) and membrane (M) fractions of these strains were subjected to SDS-PAGE on a 12.5% polyacrylamide gel, followed by immunoblot analysis using anti-OPH specific antibodies. OPH was targeted to the membrane in <i>E. coli</i> GS027 cells co-expressing ExbD<sup>N6xHis</sup> + preOPH (Panel A), ExbD<sup>N6xHis</sup> + preOPH<sup>D301A</sup> (Panel B), ExbD<sup>N6xHis</sup> + preOPH<sup>D301N</sup> (Panel C). The preOPH<sup>K82A</sup> failed to target membrane in <i>E. coli</i> GS027 cells even in the presence of ExbD<sup>N6xHis</sup> (Panel D). Lane MM indicates the mobility pattern of protein molecular weight markers obtained from a strained gel. The lower OPH specific signals indicate degradation products of OPH.

Figure 10: Lowered iron uptake and reduced growth in Δopd strain <i>S. wildii</i> DS010.

Panel A shows the reduced growth of Δopd strain <i>S. wildii</i> DS010 compared to the wild type <i>S. wildii</i> in minimal medium supplemented with 0.02 μg Fe / mL (iron-limiting conditions). The inability of the mutant strain to acquire iron (<sup>55</sup>Fe) is shown in panel B. The <i>S. wildii</i> DS010 cells were incubated with the radiolabeled ferric-enterobactin for 2h at 37°C before measuring intracellular <sup>55</sup>Fe.
Fig. 2

A

**OPH-desferritenter孝in**

![Graph showing response units (RU) vs. time (seconds) for different concentrations of OPH-desferritenter孝in.](image)

B

**OPH-Ferric-enteroxin**

![Graph showing response units (RU) vs. time (seconds) for different concentrations of OPH-Ferric-enteroxin.](image)

C

**OPH-desferritenter孝in**

![Graph showing response units (RU) vs. concentration (μM) for OPH-desferritenter孝in.](image)

D

**OPH-Ferric-enteroxin**

![Graph showing response units (RU) vs. concentration (μM) for OPH-Ferric-enteroxin.](image)

E

**OPH-desferritenter孝in**

![Graph showing fluorescence intensity vs. wavelength for OPH-desferritenter孝in.](image)

F

**OPH-Ferric-enteroxin**

![Graph showing fluorescence intensity vs. wavelength for OPH-Ferric-enteroxin.](image)
Fig. 4
Fig. 5
Fig. 6
Fig. 8

| A | ExbD<sup>N<sub>60Dls</sub></sup> - OPH Interactions | ExbD<sup>N<sub>60Dls</sub></sup> - OPH<sup>D<sub>96N</sub></sup>A Interactions |
|---|---|---|
| | Coexpression | Ind. Expression | Coexpression | Ind. Expression |
| | CL<sup>C</sup> | FT | W | E<sup>F</sup> | E<sup>O</sup> | CL<sup>O</sup> | CL<sup>E</sup> | CL<sup>C</sup> | FT | W | E<sup>F</sup> | E<sup>O</sup> | CL<sup>O</sup> | CL<sup>E</sup> |
| I | | | | | | | | | | | | | |
| II | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Anti-His | Anti-OPH |

| B | ExbD<sup>N<sub>60Dls</sub></sup> - OPH Interactions | ExbD<sup>N<sub>60Dls</sub></sup> - OPH<sup>D<sub>96N</sub></sup> Interactions |
|---|---|---|
| | Coexpression | Ind. Expression | Coexpression | Ind. Expression |
| | CL<sup>C</sup> | FT | W | E<sup>F</sup> | E<sup>O</sup> | CL<sup>O</sup> | CL<sup>E</sup> | CL<sup>C</sup> | FT | W | E<sup>F</sup> | E<sup>O</sup> | CL<sup>O</sup> | CL<sup>E</sup> |
| I | | | | | | | | | | | | | |
| II | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Anti-His | Anti-OPH |

| C | ExbD<sup>N<sub>60Dls</sub></sup> - OPH Interactions | ExbD<sup>N<sub>60Dls</sub></sup> - OPH<sup>D<sub>96N</sub></sup>E Interactions |
|---|---|---|
| | Coexpression | Ind. Expression | Coexpression | Ind. Expression |
| | CL<sup>C</sup> | FT | W | E<sup>F</sup> | E<sup>O</sup> | CL<sup>O</sup> | CL<sup>E</sup> | CL<sup>C</sup> | FT | W | E<sup>F</sup> | E<sup>O</sup> | CL<sup>O</sup> | CL<sup>E</sup> |
| I | | | | | | | | | | | | | |
| II | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Anti-His | Anti-OPH |

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**A**

**OPH^{D301A}-Ferric-enterobactin**

Response Units (RU) vs. Time (seconds)

**B**

**OPH^{D301N}-Ferric-enterobactin**

Response Units (RU) vs. Time (seconds)

**C**

**OPH^{D301A}-Ferric-enterobactin**

Equation: \[ y = a + bx \]
Plot: Concentration vs RU
Intercept: \( \text{Intercept} = 4.68846 \pm 2.8794 \)
Slope: \( \text{Slope} = 2.06692 \pm 0.06109 \)

**D**

**OPH^{D301N}-Ferric-enterobactin**

Equation: \[ y = a + bx \]
Plot: Concentration vs RU
Intercept: \( \text{Intercept} = -4.29943 \pm 2.70566 \)
Slope: \( \text{Slope} = 2.05557 \pm 0.0574 \)

**E**

**OPH^{D301A}-Ferric-enterobactin**

\[ K_D = 5.9 \times 10^{-6} \pm 4.8 \times 10^{-10} \text{ M} \]

**F**

**OPH^{D301N}-Ferric-enterobactin**

\[ K_D = 1.1 \times 10^{-6} \pm 5.3 \times 10^{-11} \text{ M} \]
