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Yersinia ruckeri YRB periplasmic binding protein YiuA selectively recognizes a Fe(III)-mono-catecholate siderophore

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The marine pathogen *Yersinia ruckeri* synthesizes the tri-catecholate siderophore ruckerbactin, Rb, (DHB-^{L}Arg-^{L}Ser)$_3$, to acquire iron during infection. Its biosynthetic gene cluster encodes a single periplasmic binding protein, RupB, which surprisingly does not bind Fe(III)-Rb nor the Fe(III) complexes of its hydrolysis products, the di- and mono-catecholate siderophores Rb_{DC} and Rb_{MC}, with biologically relevant affinities. Instead, the periplasmic binding protein YiuA, encoded in a different region of the chromosome, binds the 1:2 Fe(III) complex of the mono-catecholate Rb_{MC}, Fe(III)-$(Rb_{MC})_2$. YiuA is the first periplasmic binding protein (PBP) to selectively recognize a mono-catecholate siderophore, the structural basis of which was illuminated through X-ray crystallography of YiuA bound to Fe(III)-$(Rb_{MC})_2$.

Bacteria have evolved multiple strategies to cope with iron starvation, including the production of siderophores, chelators with high affinity for Fe(III). More than 600 siderophore structures are known, encompassing remarkable structural variability.[1, 2] Competition within microbial communities promotes siderophore diversity, enabling bacteria to secure iron by producing molecules inaccessible to their rivals,[3] and raising the broader question of which structural features confer siderophore privacy. Biosynthetic gene clusters (BGCs) for tri-catechol siderophores framed on a tri-^{L}Ser scaffold and distinguished by the presence of the D- and L-cationic amino acids (CAA) of Arg, Lys and Orn are encoded in microbial genomes, and are discoverable through genome mining.[4, 5] Within this suite of siderophores, ruckerbactin, (DHB-^{L}Arg-^{L}Ser)$_3$, produced by *Yersinia ruckeri* YRB, and trivanchrobactin, (DHB-^{D}Arg-^{L}Ser)$_3$, produced by *Vibrio campbellii* DS40M4, are diastereomers, differing only by the presence of ^{L}Arg or ^{D}Arg (DHB is dihydroxybenzamide; Fig. 1A). Both *Y. ruckeri* and *V. campbellii* are marine pathogens of notable consequence.[6, 7] *Y. ruckeri* is the causative agent of enteric red mouth disease in salmonids,[8] and *V. campbellii* inflicts luminous vibriosis.[9] These microbes can inhabit the same environments; thus, they are potentially exposed to each other's siderophores.

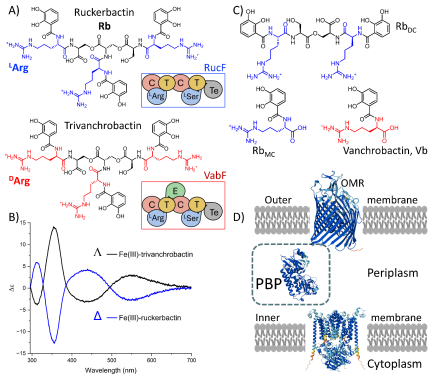

Fig. 1: Ruckerbactin and Trivanchrobactin Diastereomers: (DHB-$^{L/D}$Arg-^{L}Ser)$_3$ tri-catechol siderophores encoded in microbial genomes. **A)** Structures of ruckerbactin (with ^{L}Arg) and trivanchrobactin (with ^{D}Arg) with respective RucF and VabF NRPSs (C, condensation domain; T, thiolation domain; E, epimerization domain; Te thioesterase domain). **B)** ECD spectra of Δ Fe(III)-ruckerbactin and Λ Fe(III)-trivanchrobactin (100 μM Fe-L, 20 mM bis-tris pH 7.0). **C)** Hydrolysis products Rb_{DC} and Rb_{MC}, and vanchrobactin (Vb).[10] Hydrolysis of the Ser esters can occur spontaneously, and presumably *via* esterase activity.[11] **D)** Gram-negative bacterium showing the periplasmic binding protein (PBP) within the periplasm, which shuttles an Fe(III)-siderophore from the outer membrane receptor (OMR) protein to the inner membrane transporter.

The Fe(III) complexes of ruckerbactin and trivanchrobactin are enantiomeric at the Fe(III) coordination site, which is reflected in the electronic circular dichroism (ECD) spectra with Δ-configured Fe(III)-ruckerbactin and Λ-configured Fe(III)-trivanchrobactin (Fig. 1B). Under iron restricted conditions, a

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mutant of *V. campbellii* DS40M4 deficient in the biosynthesis of DHB grows on its native Λ-Fe(III)-trivanchrobactin siderophore, but not on Δ-Fe(III)-ruckerbactin, highlighting stereochemistry as a key recognition component of Fe(III)-siderophore uptake.[12, 13] While chiral selectivity at the OMR has not been directly investigated (Fig. 1D), the PBP FvtB (*V. campbellii*) involved in the periplasmic transport of trivanchrobactin is selective for Λ-Fe(III)-trivanchrobactin, solely, and does not bind Δ-Fe(III)-ruckerbactin.[12]

Ruckerbactin plays a central role in *Y. ruckeri's* ability to infect its host;[6] however, Fe(III)-ruckerbactin uptake has not been investigated. *Y. ruckeri* YRB encodes two genes for siderophore PBPs in its genome, of which *rupB* resides within the ruckerbactin BGC and *yiuA* exists 625 kb away from this BGC. Only YiuA is upregulated in low iron conditions.[14] Whether the selectivity of the *Y. ruckeri* PBP responsible for Fe(III)-ruckerbactin uptake mirrors the selectivity of FvtB in *V. campbellii* is not known.

Herein we report the unprecedented selectivity of the two siderophore PBPs RupB and YiuA in *Y. ruckeri* YRB. Surprisingly, RupB in the ruckerbactin BGC does not display biologically relevant affinity for the Fe(III) ruckerbactin siderophores. Instead, YiuA is the functional PBP, with selectivity for Fe(III) coordinated by two mono-catechol siderophores (Rb_{MC}; Fig. 1C), *i.e.*, Fe(III)-$(Rb_{MC})_2$, as the Λ stereoisomer. The high selectivity of YiuA towards Fe(III) complexes of the mono-catechol siderophore over the di-catechol and tri-catechol siderophores was unexpected, as this is the first case of a siderophore PBP which selectively binds an Fe(III) complex of a mono-catechol siderophore. The difference between the Fe(III)-$(Rb_{MC})_2$ and the Fe(III)-(Rb_{DC}) complexes is subtle, with only the serine ester in the backbone of Rb_{DC} setting the complexes apart. The structural basis for the intriguing selectivity of YiuA is revealed in the crystal structure of YiuA bound to Fe(III)-$(Rb_{MC})_2$, where the direct coordination of a histidine and tyrosine residue from the protein completes of the hexacoordinate environment of Fe(III).

The ruckerbactin biosynthetic gene cluster (BGC) contains one gene encoding a PBP, *rupB*, which was proposed to bind Fe(III)-Rb.[6] We therefore examined binding of Fe(III) complexes of Rb and its hydrolysis products Rb_{DC} and Rb_{MC} to RupB through its intrinsic fluorescence quenching. To our surprise, RupB does not bind Fe(III)-Rb nor the Fe(III) complexes of the hydrolysis fragments with physiologically relevant K_d values (Fig. 2A). The K_ds of Fe(III)-Rb and Fe(III)-(Rb_{DC}) are 2.2 ± 0.1 μM and 4.1 ± 0.2 μM, respectively. The Fe(III) complex of Rb_{MC} displays the weakest affinity for RupB, and within the concentration range tested, a K_d could not be determined reliably. These micromolar or higher K_d values are in stark contrast to the low nanomolar K_d values for the catechol siderophore PBPs, FepB with Fe(III)-enterobactin,[15] and CeuE and PiuA with Fe(III)-(di-DHBS), which is the tetradentate bis catecholate hydrolysis fragment of enterobactin.[16, 17] The low affinity of RupB towards the Fe(III) complexes of the ruckerbactin compounds (Fig. 2A), indicates that RupB is not the cognate PBP for any of the endogenous siderophores in *Y. ruckeri.*

In response to iron starvation, *Y. ruckeri* upregulates the expression of the non-ribosomal peptide synthetase (NRPS) RucF, responsible for the biosynthesis of ruckerbactin, as well as the enzymes involved in DHB biosynthesis.[14] The only siderophore PBP reported to increase expression is YiuA.[14] The genes *yiuA* (locus tag: BD65_762) and *rupB* (BD65_1342) are located in entirely different regions of the *Y. ruckeri* YRB chromosome, approximately 625 kb apart.

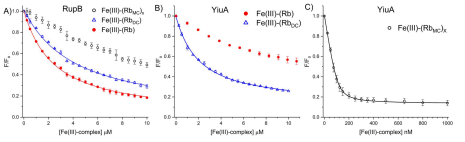

Fig. 2. Substrate selectivity of the PBPs RupB and YiuA. **A**) Fluorescence quenching of RupB by Fe(III)-ruckerbactin compounds. The individual datasets for the binding experiments are reported in Fig. S1. At a ratio of 1:4 Fe(III):Rb_{MC}, a mixture of Fe(III)-$(Rb_{MC})_3$ and Fe(III)-$(Rb_{MC})_2$ is present in solution. **B**) Fluorescence quenching of YiuA by Fe(III)-Rb and Fe(III)-(Rb_{DC}). **C**) Fluorescence quenching of YiuA by Fe(III)-$(Rb_{MC})_2$. The only Fe(III) complex of the ruckerbactin catecholate compounds that YiuA binds with high affinity is that of the 2:1 complex of Rb_{MC} and Fe(III), *vide infra* (Fig. 3). The individual binding data sets are found in Fig. S2.

YiuA also displays a low affinity for Fe(III)-Rb and it was not possible to obtain a reliable K_d in the concentration range tested (Fig. 2B). The complex Fe(III)-(Rb_{DC}) binds to YiuA with higher affinity (K_d 1.9 ± 0.1 μM) than that of Fe(III)-Rb, however the affinity remains outside the range of biological relevance (Fig. 2B). Remarkably, YiuA displays a high affinity for an Fe(III) complex of the mono-catechol Rb_{MC}, with an apparent K_d 9.2 ± 0.7 nM (Fig. 2C). YiuA also binds an Fe(III) complex of the mono-catechol siderophore Vb (the diastereomer with ^{D}Arg) with high affinity K_d 38.5 ± 2.8 nM (Fig. S2). Although YiuA favors the complex with the ^{L}Arg ligand over the ^{D}Arg ligand, both complexes bind to YiuA with nanomolar affinity, underscoring the protein's flexibility to the chirality of Arg.

To determine which species of Fe(III)-$(Rb_{MC})_x$ (i.e., x = 1, 2 or 3) binds to YiuA, the tris-catechol complexes, Fe(III)-$(Rb_{MC})_3$ with ^{L}Arg, and Fe(III)-$(Vb)_3$, with ^{D}Arg, were titrated with YiuA and monitored by UV-Vis spectroscopy (Fig. 3A and 3B). Addition of YiuA to the tris catechol complexes Fe(III)-$(Rb_{MC})_3$ (λ_{max} 495 nm; ε 5,616 M^{-1} cm^{-1}) and Fe(III)-$(Vb)_3$ (λ_{max} 496 nm; ε 5,300 M^{-1} cm^{-1}), induces a batho- and hypo-chromic shift with an isosbestic point at 560 nm and 561 nm for the complexes with Rb_{MC} and Vb, respectively. The spectral changes indicate the tris-catechol complexes are converted to a single new species. After the addition of 1.2 equivalents of YiuA to Fe(III) no further change is observed in the UV-Vis spectra. At this point the molar extinction coefficients at the λ_{max} of the ligand-to-metal charge transfer (LMCT) bands are ε 3,518 M^{-1} cm^{-1} at λ_{552} and ε 3,425 M^{-1} cm^{-1} at λ_{547} for Fe(III) complexes with Rb_{MC} and Vb, respectively. These molar extinction coefficients are approximately two thirds of the value of the tris catechol complexes and diagnostic of bis-catechol Fe(III) complexes.[18] The titrations establish YiuA has a strong preference towards the Fe(III)-$(Rb_{MC})_2$ and Fe(III)-$(Vb)_2$ bis-catechol complexes. Thus, YiuA binding readily displaces one of the catechol ligands, regardless of the chirality of the cationic amino acid, when

presented with the tris-complexes, Fe(III)-$(Rb_{MC})_3$ and Fe(III)-$(Vb)_3$. Compared to PiuA, which does not discriminate between the Fe(III) complex of one di-catechol versus two mono-catechol siderophores of the enterobactin hydrolysis products,[17] the selectivity of YiuA is distinct as it displays a high preference for the Fe(III) complex with two mono-catechol siderophores.

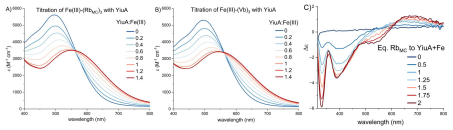

Fig. 3. Spectroscopic characterization of YiuA-bound Fe(III) complexes of Rb_{MC} and Vb. **A**) spectrophotometric titration of Fe(III)-$(Rb_{MC})_3$ with YiuA; **B**) spectrophotometric titration of Fe(III)-$(Vb)_3$ with YiuA. **C**) ECD titration of YiuA and Fe(III) with Rb_{MC}. Addition of 2 eq. of Rb_{MC} to YiuA-Fe leads to the Λ-configured Fe(III)-$(Rb_{MC})_2$. The experimental details are found in the Methods section of the SI.

Hexa-coordinate complexes with catechol ligands can form chiral bis- and tris-catechol complexes (Λ and Δ isomers), with Fe(III)-enterobactin adopting the Δ configuration.[19] Upon substrate binding, however, siderophore PBPs are known to induce Λ chirality at the Fe(III) center.[15] To assess the chirality of YiuA-bound Fe(III)-$(Rb_{MC})_2$, a solution of equimolar YiuA and Fe(III) was titrated with Rb_{MC}. The ECD spectra from the titration (Fig. 3C) produced a positive band at ~650 nm and a negative band at ~550 nm, the signature of a Λ-configured bis-catechol complex.[15] Thus, the ECD spectra demonstrate that YiuA binds Fe(III)-$(Rb_{MC})_2$ in the Λ-configured stereoisomer.

Having discovered the preference of YiuA for Λ-Fe(III)-$(Rb_{MC})_2$, we sought to investigate the structure of YiuA with Fe(III)-$(Rb_{MC})_2$ by X-ray crystallography. The structure of YiuA was determined in two crystal forms, *i.e.*, one with YiuA-Fe-$(Rb_{MC})_2$, and one with YiuA bound to iron without the siderophore ligand, YiuA-Fe. Each crystal form provides different information on loop mobility and ligand binding as described below.

Following building and refinement of protein atoms and water molecules in YiuA-Fe-$(Rb_{MC})_2$, clear electron density was observed in the omit maps in the iron binding site that was refined as two molecules of Rb_{MC} coordinating to one iron center, as shown in Fig. 4A for chain C. Four of the coordination sites of the distorted octahedral iron center are occupied by the two Rb_{MC} ligands, with the two DHB oxygen donors in *ortho*- and *meta*-positions, respectively, occupying adjacent coordination sites (*cis, cis* geometry). Consequently, the remaining two coordination sites are inequivalent and filled by the oxygen donor of Tyr296 and the nitrogen donor of His102 (Fig. 4A-C). The Fe(III)-$(Rb_{MC})_2$ complex is bound with the metal center in the Λ-configuration (Fig. 4B), in alignment with the solution stereochemistry (Fig 3C).

YiuA is selective for Fe(III) complexes of mono-catechol siderophores (i.e., Rb_{MC}), although its binding pocket resembles that of siderophore PBPs which bind Fe(III) di-catechol siderophores (e.g., di-DHBS).[14, 20, 21] For example, YiuA contains a basic triad of positively charged Arg residues that attract the negatively charged Fe(III)-catecholate complexes. In YiuA Arg26 and Arg127 donate H-bonds to DHB oxygen donors, Fig. 4A-C. Furthermore, YiuA binds Fe(III)-$(Rb_{MC})_2$ in the Λ-configured *cis, cis*- isomer (Fig. 4C), consistent with the Fe(III)-di-catechol siderophores.[14, 20, 21]

The difference from the previously reported crystal structures[14, 20, 21] is the orientation of the Ser residue in each Rb_{MC} which rotate outwards and face away from each other (Fig. 4A-C). Few specific interactions between the Ser residues with the protein were observed. One accepts an H-bond from the backbone of Glu190, while the other is involved in two H-bonding interactions with a sulfate anion. In turn, the Arg arms of the two Rb_{MC} ligands point into the solvent-exposed space, where they interact with the same sulfate anion originating from the buffer (Fig. 4D).

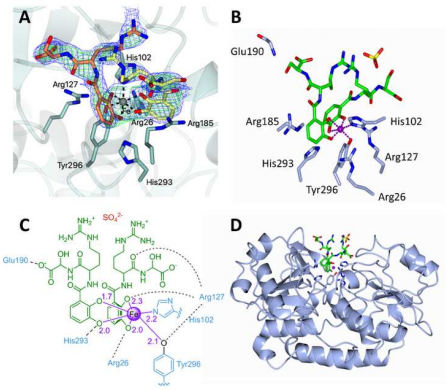

Fig. 4. **A**) Structure of YiuA-Fe-$(RbMC)_2$ showing binding of iron and two molecules of Rb_{MC} with carbon atoms in yellow and coral. Selected interactions are shown with black dashed lines. Electron density is from subunit 'G' and represents the 2Fo-Fc and Fo-Fc omit maps obtained prior to ligand atom refinement and are shown at levels of 1σ and 3 σ in blue and green respectively. **B**) Close up view of the ligand binding pocket. Rb_{MC}, colored by atom type with carbon atoms in green; Fe in purple, with its contacts in purple; and the neighboring side chains as cylinders with the carbon atoms in ice blue. **C**) Schematic diagram of key protein-ligand contacts. Fe-donor atom distances are shown in purple. H-bonds are shown in black. **D**) Structure of YiuA-Fe-$(Rb_{MC})_2$ showing the mono catechol ligands (carbon atoms in green) in the binding pocket.

Overall, the characteristic structural features of the YiuA binding pocket are well suited to the binding of Fe(III) complexes with two rather than three coordinated catecholate units. The discrimination towards the Fe(III)-complex of two mono catechol siderophores over that of the Fe(III) complex of one di-catechol siderophore stems from the rotational constraints that the serine ester imposes in the latter. In an Fe(III) complex with Rb_{DC}, the ester would force the Ser resides to point towards each other, rather than outwards as seen in the crystal structure with Fe(III)-$(Rb_{MC})_2$ (Figs. 4A and 4B). Furthermore, hydrogen bonding with Glu190 is not possible in the complex with Fe(III)-(Rb_{DC}) because of the inward facing orientation of the serine residues. The scarcity of specific interactions between the Ser and Arg arms of the siderophore ligands with the protein, however, is consistent with YiuA's

observed promiscuity towards binding both Fe(III)-$(Rb_{MC})_2$ and Fe(III)-$(Vb)_2$.

A crystal structure of YiuA bound with Fe(III) but lacking ligand electron density was obtained in the initial screening processes (YiuA-Fe). Compared to structures of YiuA-Fe-$(Rb_{MC})_2$ the Ser100-Glu109 loop in YiuA-Fe is in an extended state protruding away from the binding pocket. The structure of YiuA-Fe is thus profoundly different since His102 harbored in the Ser100-Glu109 loop is relocated away from the iron binding site, contrasting its position in the YiuA-Fe-$(Rb_{MC})_2$. An overlay of the two crystal forms highlights the flexible nature of the loop containing His102 (Fig. S3).

Since YiuA is selective towards the complex with two mono-catechol fragments of ruckerbactin, we investigated whether *Y. ruckeri* YRB possesses a periplasmic esterase, as this could enable utilization of Fe(III)-ruckerbactin. BLASTp did not reveal any esterase homologs of Cee,[22] PfeE,[23] or IroE,[24] however, a periplasmic esterase significantly different from known Ser esterases[22-24] could be operative. Additionally, the Ser esters in ruckerbactin and Rb_{DC} are prone to hydrolyze at neutral pH, ultimately yielding Rb_{MC}. Thus, while the fate of ruckerbactin remains unknown for now, the mono catechol hydrolysis product is expected to exist during infection.

In conclusion, the only PBP encoded in the ruckerbactin BGC, RupB, does not bind any of the Fe(III) ruckerbactin compounds with a high affinity (Fig. 2A). Instead, YiuA is the functional PBP for the 1:2 complex of Fe(III) and Rb_{MC} with a nM K_d (Fig. 2C). ECD spectroscopy and X-ray crystallography reveal the bis-catechol complex Fe(III)-$(Rb_{MC})_2$ binds to YiuA as the Λ stereoisomer (Fig. 3C, Fig. 4B). X-ray crystallography also establishes Fe(III)-$(Rb_{MC})_2$ is coordinated by Tyr296 and His102, in which the latter is located on a flexible loop (Fig. 4). In addition to the ^{L}Arg-containing Rb_{MC} siderophore, YiuA binds the 1:2 complex of Fe(III) and the ^{D}Arg-containing diastereomer Vb with high affinity, hinting the PBP may enable siderophore piracy. Whether YiuA can recognize the Fe(III)-complexes of the entire suite of mono-catechol L/D-cationic amino acid siderophores (e.g., DHB-$^{L/D}$CAA-^{L}Ser with Lys, Orn) is currently under investigation.

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Conflicts of interest

There are no conflicts to declare.

Data availability

The materials and methods and additional data that support the findings of this study are available in the supplementary material of this article.

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Deleted: Rb_{MC} and Vb belong to the larger family of diastereomeric catechol siderophores, which contain Orn and Lys as the cationic amino acid in the L and D chirality.

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