THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 46, pp. 35413–35424, November 17, 2006

Interactions between TonB from *Escherichia coli* and the Periplasmic Protein FhuD*

Received for publication, August 9, 2006 Published, JBC Papers in Press, August 23, 2006, DOI 10.1074/jbc.M607611200

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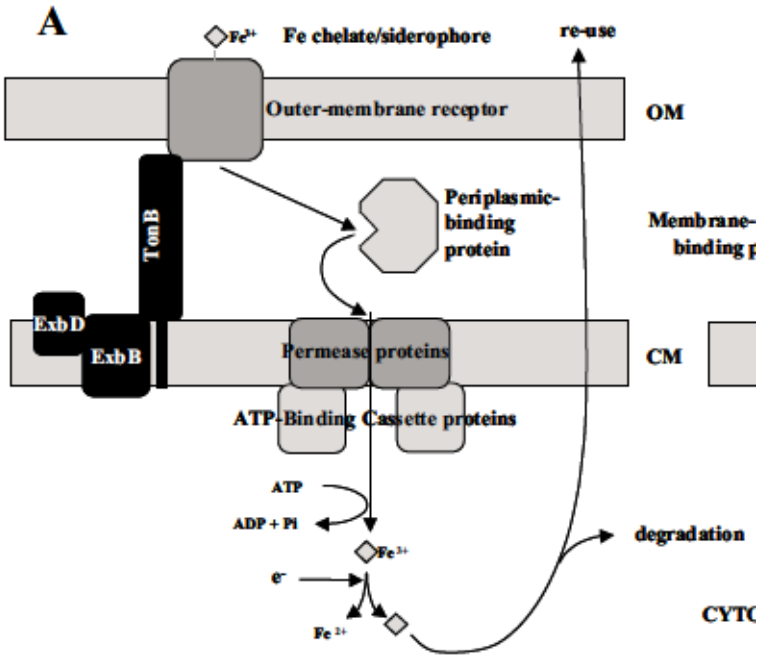
Sistema per l'internalizzazione del sideroforo ferricromo in batteri Gram-negativi
FhuA-TonB-FhuD
FhuB-FhuC



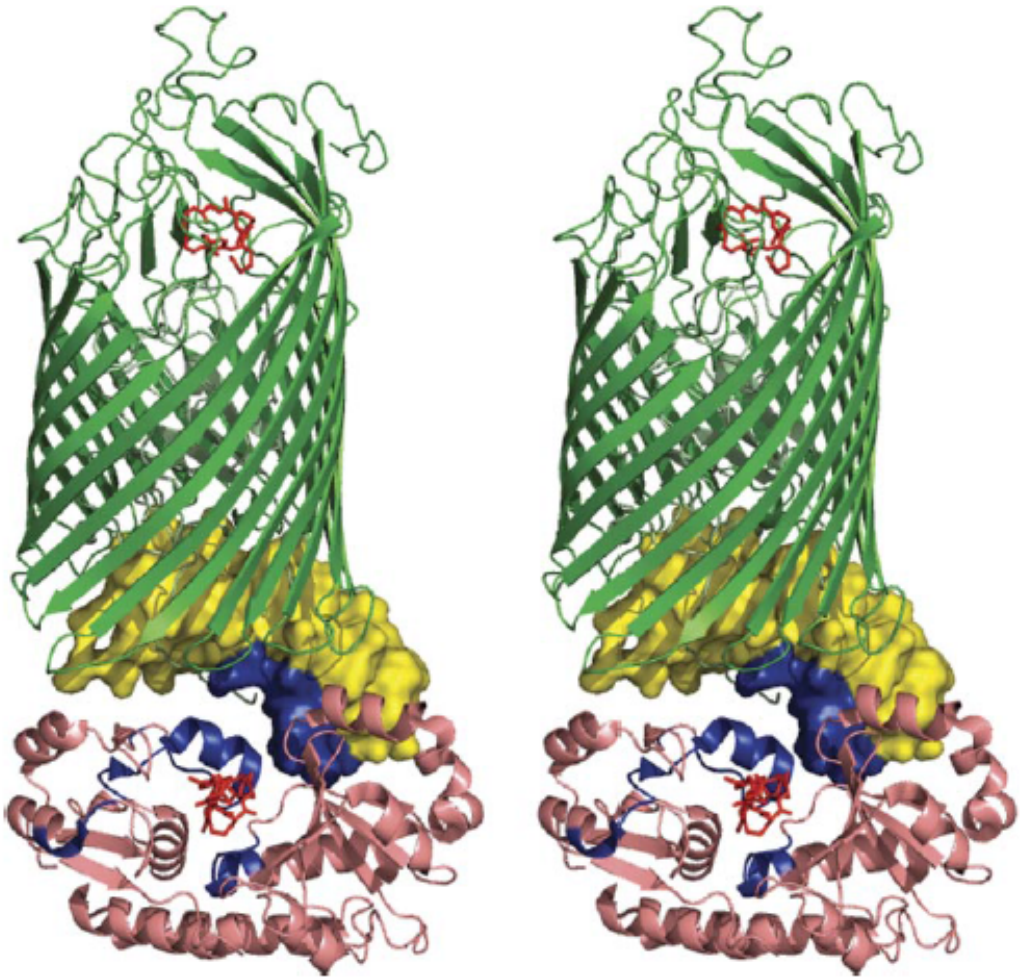


FIGURE 9. **Model of a FhuA-TonB-FhuD ternary complex.** Stereo image depicting a possible ternary complex between FhuA, TonB, and FhuD. FhuD (PDB code 1EFD) was manually docked under the TonB-FhuA crystal structure (PDB code 2GRX) using phage display-identified protein-protein interaction surfaces as docking constraints. Complementary phage display-identified surfaces are colored *blue* on both TonB (*yellow*, surface representation) and FhuD (*salmon*, ribbon representation). The orientation localizes the FhuD siderophore-binding site beneath the lumen of FhuA (*green*, ribbon representation). For clarity, a molecular surface is projected on TonB.

Obiettivo del lavoro

- Determinare gli eventi molecolari alla base della cattura del ferricromo da parte di FhuD dopo il rilascio, mediato da TonB, del sideroforo da FhuA ➔ identificare e caratterizzare l'interazione tra FhuD e il complesso TonB-FhuA
- Era già noto che:
  - La struttura di FhuA è costituita da una regione N-terminale globulare che forma un 'tappo' che si inserisce in un β-barile a 22 filamenti
  - Nel complesso TonB-FhuA la regione C-terminale di TonB contatta la regione N-terminale di FhuA e potrebbe mediare lo spostamento del 'tappo' e quindi il passaggio del sideroforo

Come fare?

- Ottenere proteine purificate
  - Espressione ricombinante in *E. coli* di FhuD e TonB wild type e mutanti FhuD T181C e TonB Cys1 con His-tag e purificazione su resina Ni-NTA
- Identificare e analizzare qualitativamente e quantitativamente le interazioni tra FhuD e TonB
  - Phage display
  - Spettroscopia di fluorescenza
  - SPR

Identificazione di regioni di interazione tra TonB e FhuD: phage display

- Libreria di peptidi a sequenza casuale esposti sulla proteina pIII del fago M13
  - Ph.D.-C7C peptidi ciclici di 7 aa con un ponte disolfuro
  - Ph.D.-12 peptidi lineari di 12 aa
- Screening su proteina immobilizzata (TonB oppure FhuD) in piastre da 96 pozzetti
- Estrazione ssDNA da fagi che si legano alla proteina e sequenziamento

Strategia per la costruzione di librerie peptidiche lineari o cicliche per phage display

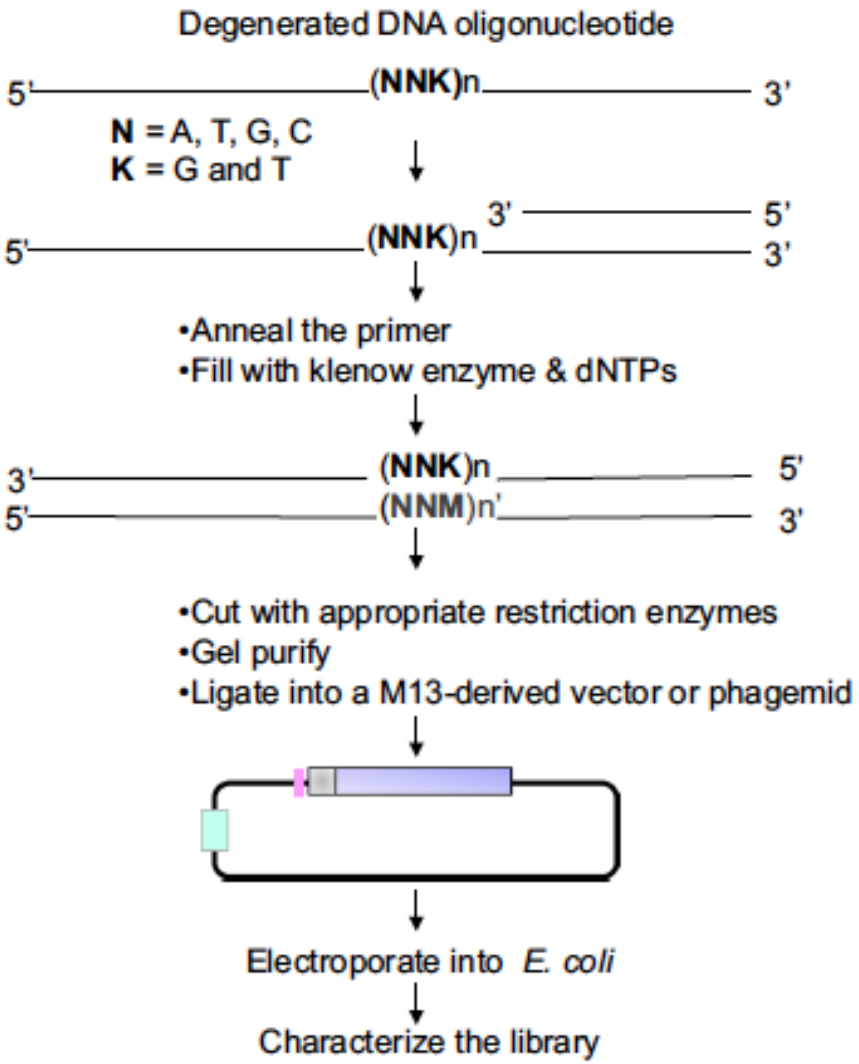


Fig. 3 Random peptide libraries. The concept of random peptide phage display is based on insertion of random oligonucleotides at the appropriate location within one of the phage coat proteins such as the N-terminus of pIII or pVIII. Each amino acid residue in the inserted peptides is randomly encoded by the degenerated codon (NNK). Prior to cloning, the random oligonucleotides are then converted into double-stranded DNAs using an annealing primer and klenow DNA polymerase. Finally, the double-stranded DNAs are digested with the appropriate restriction enzymes to produce DNA fragments that can be cloned directly into a phage vector. The letter *n* represents the number of inserted amino acid residues. Large peptide inserts up to 38 aa can be inserted into the N-terminus of pIII protein without the loss of phage infectivity or particle assembly [20]. It should be noted that the incorporation of conserved cysteine residues at the desired locations in the degenerated oligonucleotides (e.g., $C(NNK)_nC$) allows the generation of cyclic peptide libraries [21]

Librerie peptidiche phage display selezionate su TonB e mappatura delle sequenze dei peptidi sulla sequenza di FhuD

TABLE 1

RELIC/MATCH Identification of TonB affinity-selected Ph.D.-C7C peptides corresponding to FhuD sequences

Peptide[a]	Peptide match score[b]	Scoring window (residues)	Region	Alignment position[c]
PYGAALH	16	5	Loop 2	26
PYGAALH	16	5	Loop 23	245
YGGATLL	15	5	Loop 23	245
QP**AVANT**	13	5	Loop 2	28
SY**LNVMH**	13	5	Loop 23	249
TG**PLPN**R	13	4	Helix 2	43
NPTPEKR	13	4	Loop 8	78
KP**SSPPF**	12	4	Helix 2	40

[a] Residues contained in scoring window are shown in boldface type.
[b] Sum of pairwise comparisons of aligned residues within the scoring window.
[c] Position of first residue of the scoring window numbering from the N terminus of the mature protein.

TABLE 2

RELIC/MATCH Identification of TonB affinity-selected Ph.D.-12 peptides corresponding to FhuD sequences

Peptide[a]	Peptide match score[b]	Scoring window (residues)	Region	Alignment position[c]
LL**ADTTHHR**PWT	17	7	Loop 2	30
HWKH**PWGAWDTL**	16	7	Loop 2	26
KVWSL**EPPGP**AA	15	5	Helix 2	41
YSP**PSPEP**PRIK	15	5	Loop 8	78
QD**RGILVEPPR**M	14	8	Helix 2	36
D**FDVSFLSA**RMR	14	8	Loop 23	244
KLWELNPPQVRT	14	7	Helix 2	37
SP**APTNNY**TYRL	14	6	Loop 2	30
TQP**LGLLP**SRHL	14	5	Loop 2	22
QTALI**TIHHSLT**	13	6	Loop 2	32
YGNSLPPRLGPP	13	5	Loop 23	245
LWA**KLWV**VPERA	12	5	Helix 2	36
SANLSWRESWPT	12	5	Loop 23	246

[a] Residues contained in scoring window are shown in boldface type.
[b] Sum of pairwise comparisons of aligned residues within the scoring window.
[c] Position of first residue of the scoring window numbering from the N terminus of the mature protein.

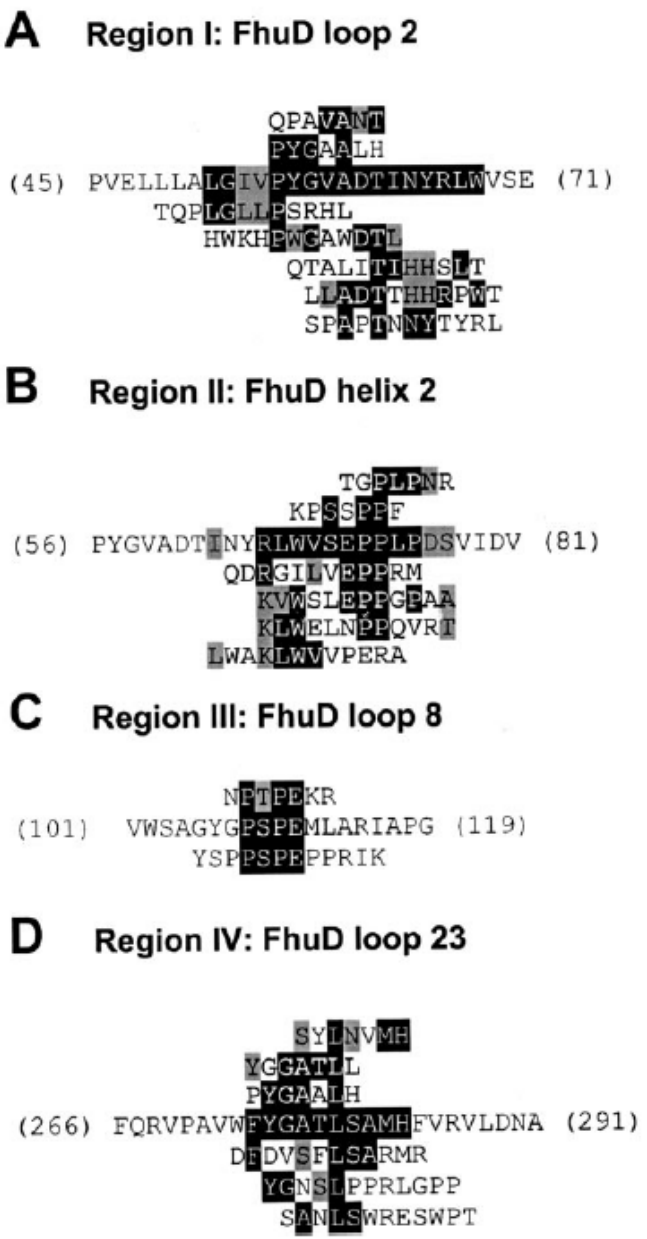


FIGURE 1. **Alignments of TonB affinity-selected peptides to FhuD as identified by RELIC/MATCH.** *A, region I,* FhuD loop 2; *B, region II,* FhuD helix 2; *C, region III,* FhuD loop 8; *D, region IV,* FhuD loop 23. Peptides from the Ph.D.-12 library are aligned below the FhuD sequence; peptides from the Ph.D.-C7C library are aligned above the FhuD sequence. See Tables 1 and 2 for peptide match scores and window sizes. Alignment positions are highlighted according to their pairwise alignment score: +4, *black background* and *white characters*; +1, *gray background* and *dark characters*.

Posizione nella struttura di FhuD delle regioni identificate con phage display

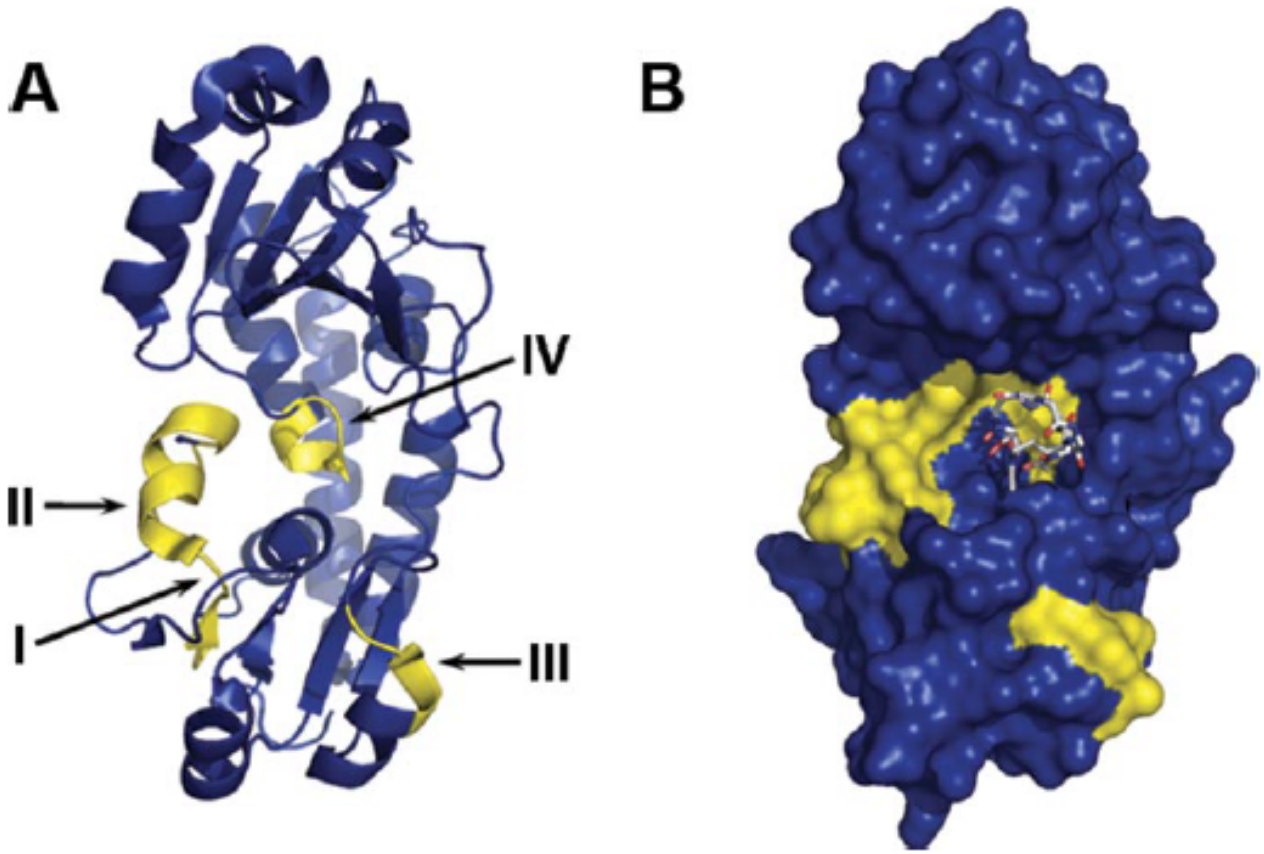


FIGURE 2. **TonB-binding regions identified by phage display mapped to FhuD (PDB code 1EFD).** *A,* ribbon representation of FhuD (*blue*) with predicted TonB-binding regions *shaded yellow.* Regions corresponding to RELIC/MATCH alignment clusters shown in Fig. 1 are indicated by roman numerals: *I,* loop 2; *II,* helix 2; *III,* loop 8; *IV,* loop 23. *B,* molecular surface representation of FhuD (*blue*) with predicted TonB-binding regions *shaded yellow.* The bound ligand gallichrome from the 1EFD structure is shown in stick representation and colored by atoms (carbon, *white*; nitrogen, *blue*; oxygen, *red*).

Librerie peptidiche phage display selezionate su FhuD e mappatura delle sequenze dei peptidi sulla sequenza di TonB

TABLE 3

RELIC/MATCH identification of FhuD affinity-selected Ph.D.-C7C peptides corresponding to TonB sequences

Peptide[a]	Peptide match score[b]	Scoring window (residues)	Region	Alignment position[c]
PAPERPQ	16	6	N-terminal	39
HASPAHN	15	6	Intermediate	121
VISAASQ	15	6	C-terminal	146
Q**SFPRQL**	14	6	C-terminal	149
NRPSSWL	14	5	Intermediate	119
TA**ENSSP**	13	5	Intermediate	124
KTSPAWI	13	5	Intermediate	127
M**TARTTS**	13	5	Intermediate	129
I**SPAQ**SS	13	4	N-terminal	39
PA**VPAKA**	12	5	N-terminal	36
HLAPAAR	12	5	Intermediate	127
KALMRTS	12	5	C-terminal	153
KPL**FHNT**	12	4	Intermediate	122
HHW**APTR**	12	4	Intermediate	126
HNM**PAQT**	12	3	N-terminal	38

[a] Residues contained in scoring window are shown in boldface type.
[b] Sum of pairwise comparisons of aligned residues within the scoring window.
[c] Position of first residue of the scoring window numbering from the N terminus of the mature protein.

TABLE 4

RELIC/MATCH identification of FhuD affinity-selected Ph.D.-12 peptides corresponding to TonB sequences

Peptide[a]	Peptide match score[b]	Scoring window (residues)	Region	Alignment position[c]
LHTPWH**LPAP**EI	16	4	N-terminal	32
KSLSRHDHIHHH	15	6	C-terminal	153
YHSPPH**TPPAPL**	14	6	Intermediate	122
SFVG**LVELP**QNL	14	5	N-terminal	31
VSRHQSWHPHDL	14	5	C-terminal	155
KTLTL**PLSNTS**K	13	6	Intermediate	119
KIMRMP**RLMTRN**	13	6	C-terminal	147
LHFPLDY**PQALG**	13	5	C-terminal	145
WHSPWSTP**PAPS**	13	4	N-terminal	31
LHWPLYTP**PASP**	12	4	N-terminal	33

[a] Residues contained in scoring window are shown in boldface type.
[b] Sum of pairwise comparisons of aligned residues within the scoring window.
[c] Position of first residue of the scoring window numbering from the N terminus of the mature protein.

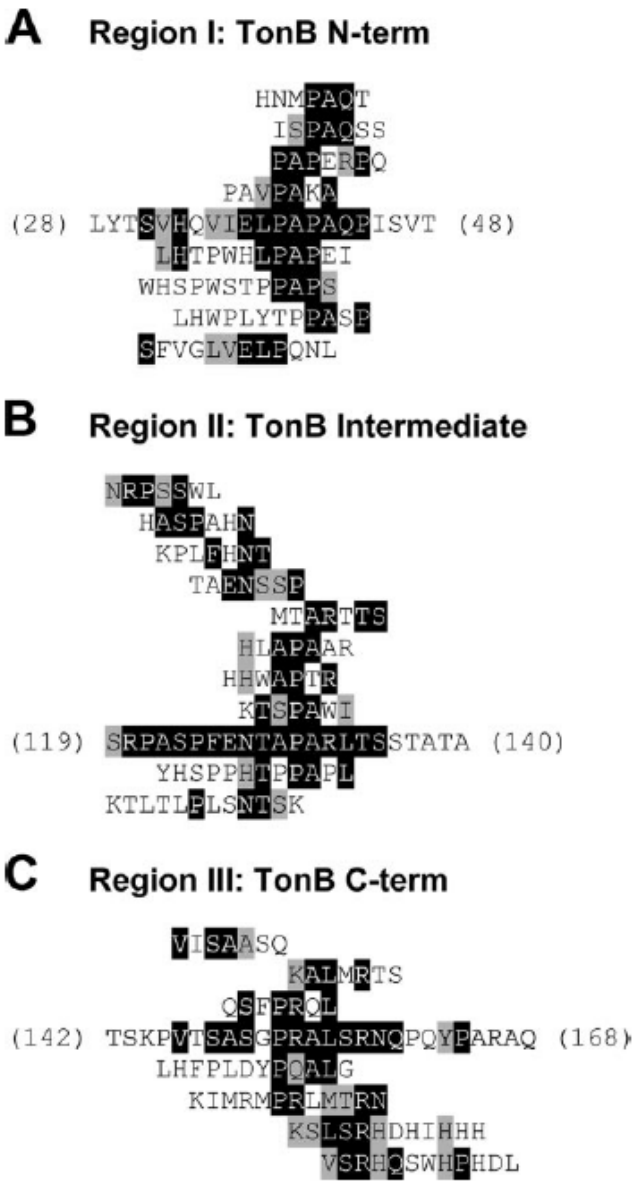


FIGURE 3. **Alignments of FhuD affinity-selected peptides to TonB.** *A*, region I, TonB N-terminal domain; *B*, region II, TonB intermediate domain; *C*, region III, TonB C-terminal domain. Peptides from the Ph.D.-12 library are aligned below the TonB sequence; peptides from the Ph.D.-C7C library are aligned above the TonB sequence. See Tables 3 and 4 for peptide match scores and window sizes. Alignment positions are highlighted according to their pairwise alignment score: +4, *black background* and *white characters*; +1, *gray background* and *dark characters*.

Posizione nella struttura della regione C-terminale di TonB delle sequenze identificate con phage display

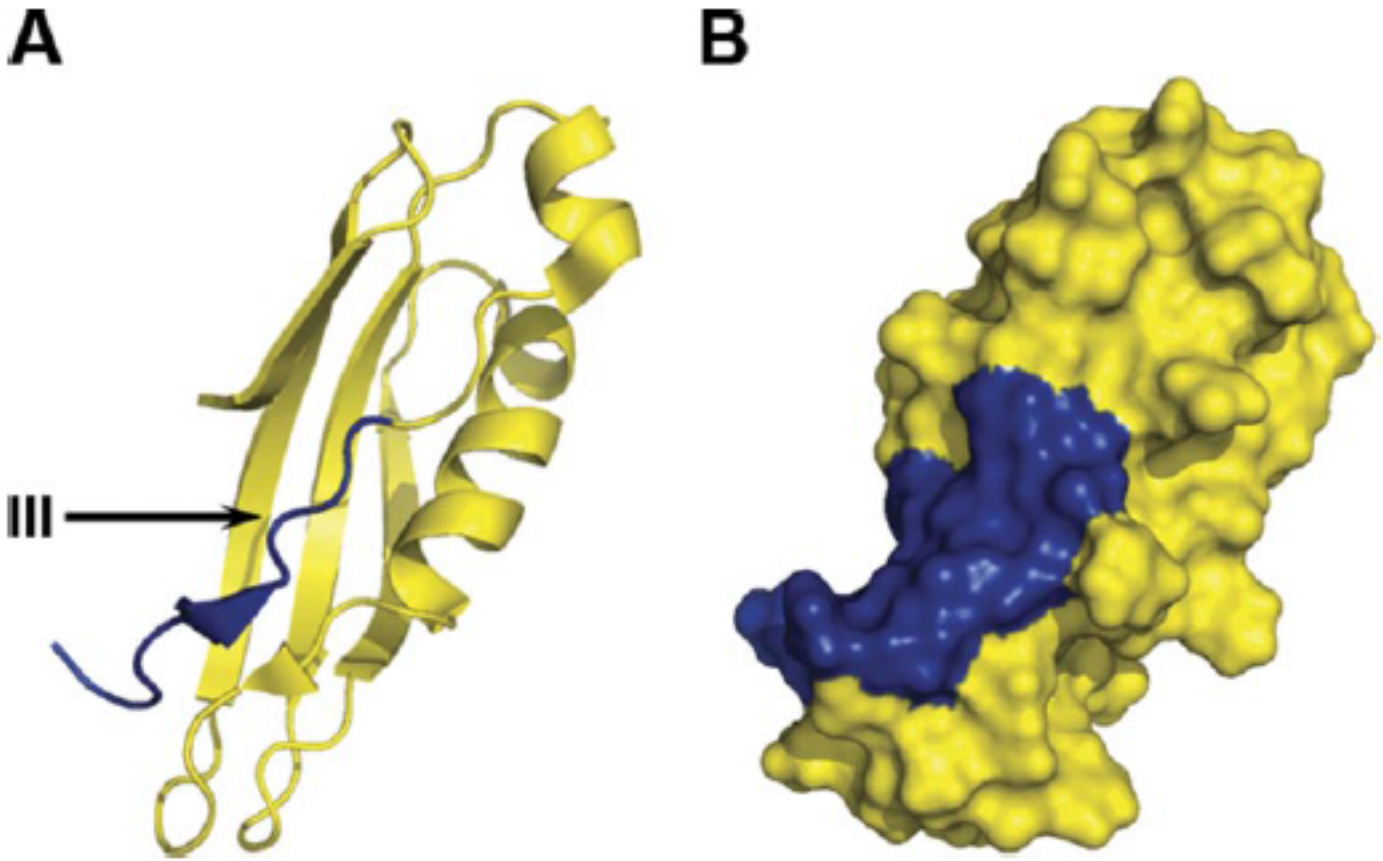


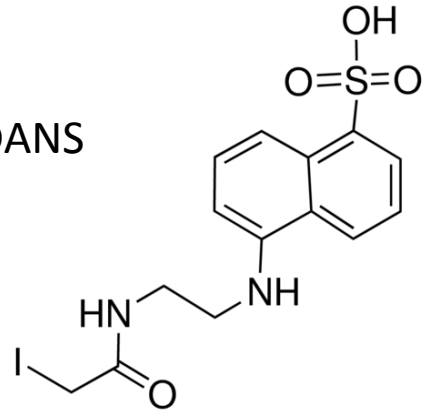
FIGURE 4. **FhuD-binding region identified by phage display mapped to TonB (PDB code 1XX3).** *A*, ribbon representation of the TonB C-terminal domain (*yellow*) with predicted FhuD-binding region III shaded *blue*. The region corresponding to RELIC/MATCH alignment cluster shown in Fig. 3*C* is indicated by the roman numeral *III*. *B*, molecular surface representation of TonB (*yellow*) with the predicted FhuD-binding region III shaded *blue*.

Analisi quantitativa dell'interazione TonB-FhuD

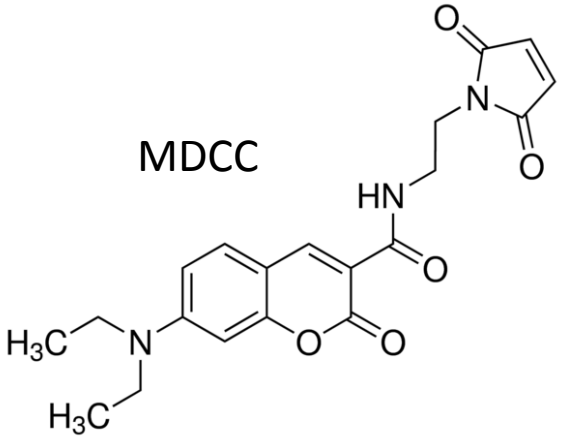
Spettroscopia di fluorescenza

- Fluorescenza intrinseca triptofano
- Marcatura FhuD T181C con sonde fluorescenti AEDANS e MDCC
- Analisi legame sideroforo ferricrocina
  - Conferma integrità del mutante
- Analisi interazione con TonB

AEDANS



MDCC



Marcatura di FhuD T181C con AEDANS o MDCC

- Incubazione con il reattivo (10 eccessi) per 4 ore a T ambiente al buio
- Aggiunta di β-mercaptoetanolo per fermare la reazione (inattivazione reattivo in eccesso)
- Dialisi per rimuovere reattivo in eccesso
- Determinazione dell'efficienza di marcatura
  - Spettroscopia di assorbimento: A_{339} AEDANS (ε_{336} 5700 M^{-1} cm^{-1}) e A_{419} MDCC (ε_{419} 50000 M^{-1} cm^{-1})
  - Dosaggio proteina (saggio di Bradford)

Analisi del quenching della fluorescenza

Fluorescenza intrinseca triptofano

λ_{exc} 280 nm λ_{em} 340 nm

FhuD 1.5 μM

Fluorescenza sonde

AEDANS λ_{exc} 336 nm λ_{em} 490 nm

MDCC λ_{exc} 419 nm λ_{em} 466 nm

FhuD 0.5 μM

FhuD:AEDANS circa 1:1

FhuD:MDCC circa 1:0.3

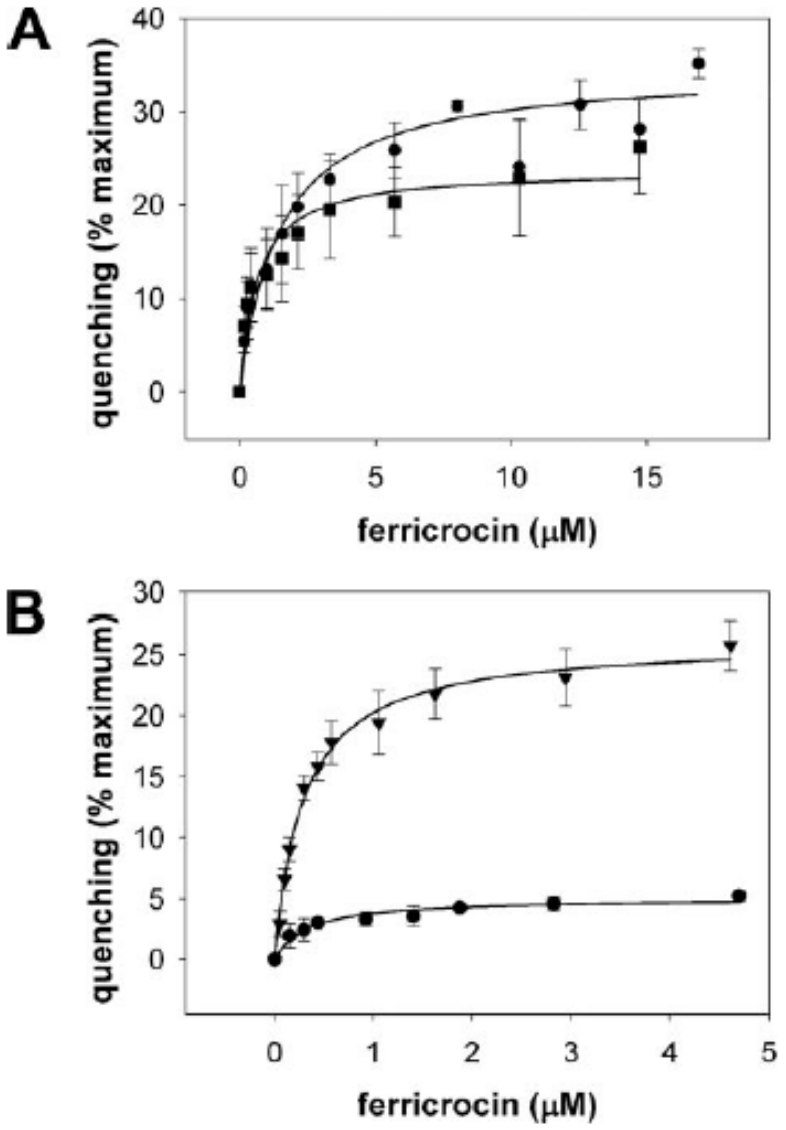


FIGURE 5. **Binding of Fcn to FhuD and to FhuD T181C.** *A*, FhuD (●) and FhuD T181C (■) were titrated with the indicated amounts of Fcn; quenching of intrinsic fluorescence is plotted as a function of Fcn concentration. *B*, binding of Fcn to AEDANS-labeled FhuD T181C (●) and to MDCC-labeled FhuD T181C (▼). Proteins were titrated with Fcn, and quenching of probe fluorescence was plotted as a function of Fcn added. *Error bars* in *A* and *B* represent the standard deviation from three independent experiments. *Lines* through data indicate best fits to a single binding site model as determined with Sigmaplot.

Determinazione della K_D per il legame di FhuD con la ferricrocina o con TonB

TABLE 6

Summary of ligand binding parameters fit to a single site saturation ligand binding model

Data are from the following equation: $y = B_{max} \times [L]/(K_D + [L])$, where [L] indicates ligand concentration; reported uncertainties represent standard errors associated with best fits to the single binding site model.

FhuD	Titration with ferricrocin			Titration with TonB		
	Maximum quench	$K_{D(app)}$	R^2	Maximum quench	$K_{D(app)}$	R^2
	%	μM		%	μM	
Wild type	33 ± 2	1.2 ± 0.2	0.8857			
T181C	24 ± 2	0.6 ± 0.2	0.7725			
T181C-AEDANS	6.9 ± 0.4	0.9 ± 0.2	0.8368	5.8 ± 0.2	0.31 ± 0.05	0.9531
T181C-AEDANS + Fcn[a]				5.9 ± 0.3	0.4 ± 0.1	0.9323
T181C-MDCC	27 ± 1	0.31 ± 0.03	0.9614	70 ± 3	0.27 ± 0.04	0.9498
T181C-MDCC + Fcn				49 ± 3	0.5 ± 0.1	0.9765

[a] Fcn is ferricrocin.

$$\Delta F = \Delta F_{\max} \frac{[fcn]}{K_D + [fcn]}$$

$$\Delta F = \Delta F_{\max} \frac{[TonB]}{K_D + [TonB]}$$

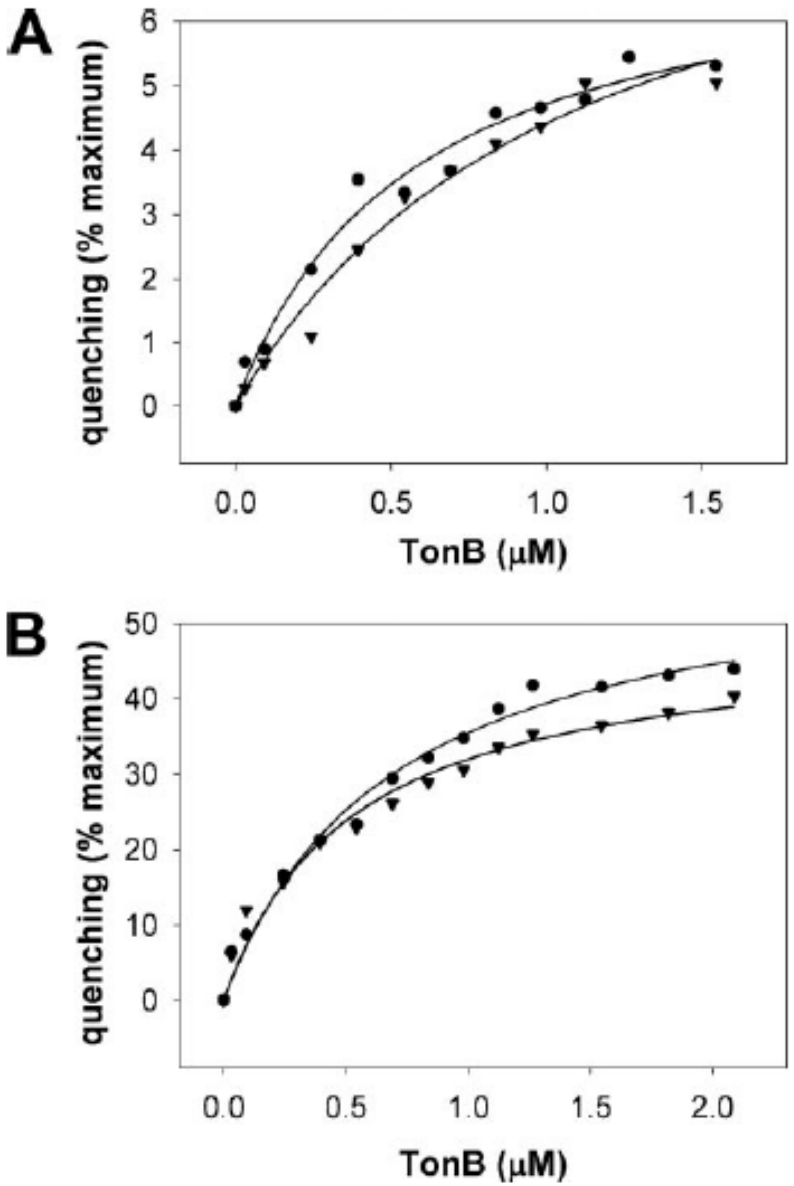


FIGURE 6. **Binding of TonB to AEDANS-labeled FhuD T181C and to MDCC-labeled FhuD T181C.** TonB was added to a solution of labeled FhuD (in the absence or presence of Fcn), and changes in extrinsic fluorescence were recorded. *A*, response upon addition of TonB to either FhuD T181C-AEDANS (●) or Fcn-bound FhuD T181C-AEDANS (▼). *B*, response upon addition of TonB to either FhuD T181C-MDCC (●) or Fcn-bound FhuD T181C-MDCC (▼). *Lines* through data indicate best fits to a single binding site model as determined with Sigmaplot. Results are representative of three experiments.

Analisi quantitativa dell'interazione TonB-FhuD

SPR

- Immobilizzazione su chip
  - TonB (immobilizzazione via -NH_2)
  - Cys-TonB (immobilizzazione via -SH)
- Interazione con FhuD
  - Effetto ferricrocina
- Complesso ternario FhuA-TonB-FhuD
  - TonB + FhuA + FhuD
  - TonB + FhuD + FhuA

Complesso TonB-FhuD

K_D 20 nM
k_{on} $2 \cdot 10^4$ M^{-1} s^{-1}
k_{off} $4 \cdot 10^{-4}$ s^{-1}

La differenza tra i valori di K_D ottenuti per SPR e fluorescenza potrebbe essere dovuta alle diverse condizioni sperimentali
Affinità TonB-FhuD intervallo 20-500 nM

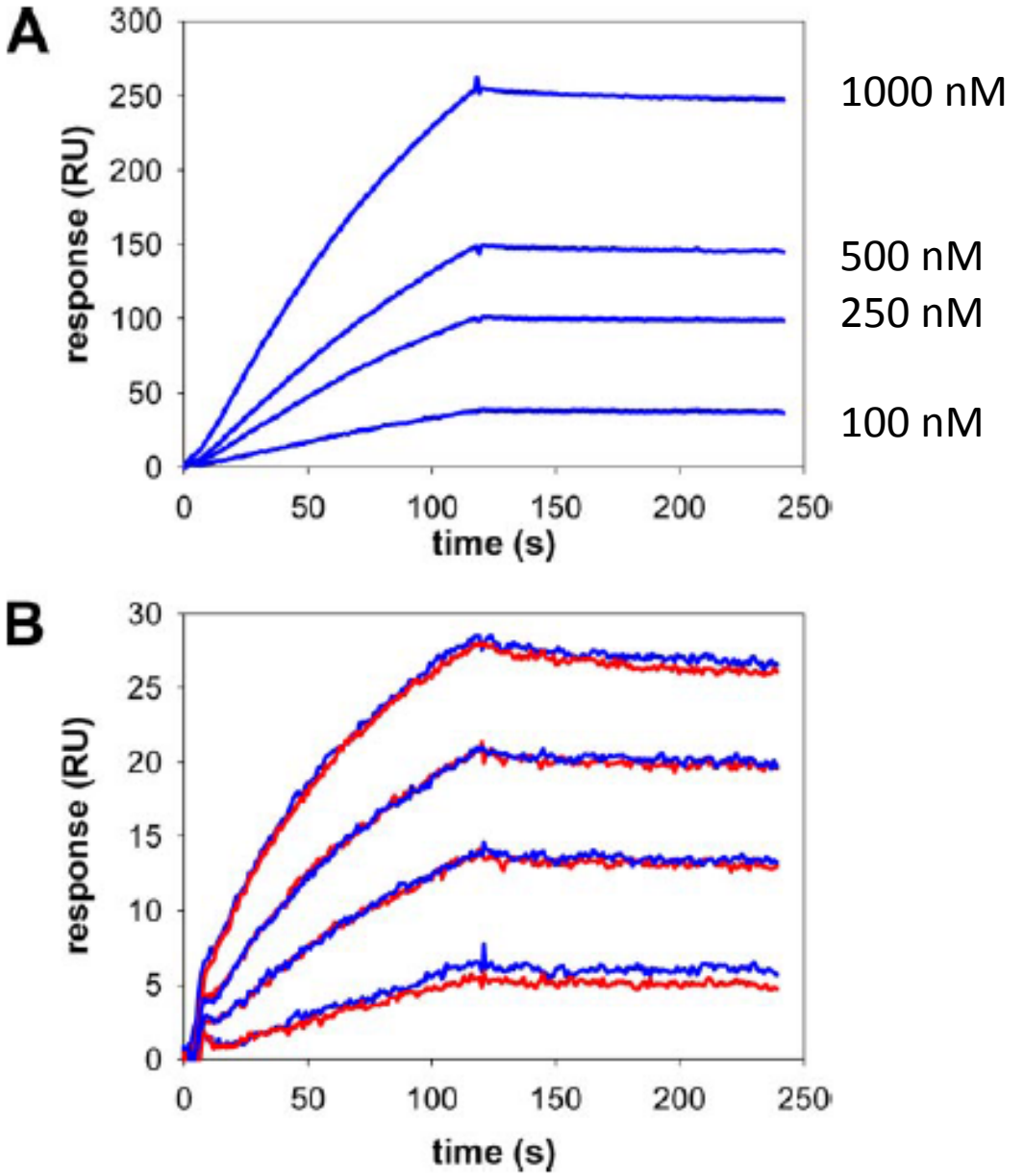


FIGURE 7. **Real time kinetics of TonB-FhuD binding interaction detected by SPR.** *A*, representative SPR sensogram for FhuD (*top to bottom*: 1000, 500, 250, and 100 nM) binding to amine-coupled TonB (250 RU) in the absence of Fcn. *B*, representative SPR sensogram for FhuD (*top to bottom*: 1000, 500, 250, and 100 nM) binding to thiol-coupled Cys-TonB (48 RU) in the absence (*blue*) or presence (*red*) of a 10-fold molar excess of Fcn.

TonB + FhuA 1 μM
lavaggio
+ FhuD 1 μM
rigenerazione

TonB + FhuD 1 μM
lavaggio
+ FhuA 1 μM
rigenerazione

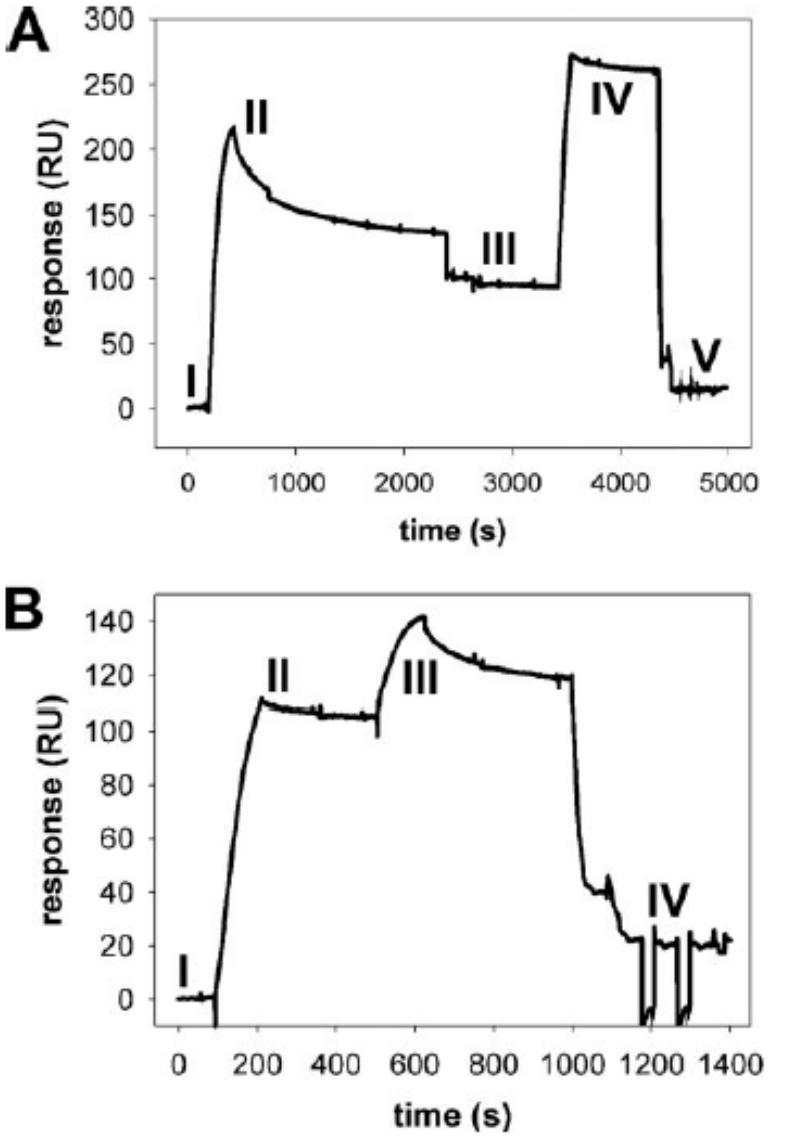


FIGURE 8. **Multicomponent SPR analysis to detect ternary complex formation between FhuA-TonB-FhuD.** *A*, SPR sensogram indicating the following: *I*, base line for buffer flowing over amine-coupled TonB (250 RU); *II*, increased signal change due to binding of FhuA (1 μM); *III*, stable FhuA-TonB complex after a 0.5 M NaCl wash; *IV*, increased signal change due to binding of FhuD (1 μM); *V*, return to base line after regeneration. *B*, SPR sensogram indicating the following: *I*, base line for buffer flowing over amine-coupled TonB (250 RU); *II*, increased signal change due to binding of FhuD (1 μM); *III*, increased signal change due to binding of FhuA (1 μM); *IV*, return to base line after regeneration.

In conclusione

- Modello strutturale dell'interazione FhuA-TonB-FhuD
- La K_D circa 1 μM di FhuD per il sideroforo suggerisce che per rendere efficiente il processo di trasporto attraverso il periplasma sia necessaria una interazione diretta transiente con TonB (K_D 20-500 nM)
- TonB agisce da 'scaffold' regolando il flusso del sideroforo (mediato da FhuA-FhuD-FhuB) dalla membrana esterna a quella interna

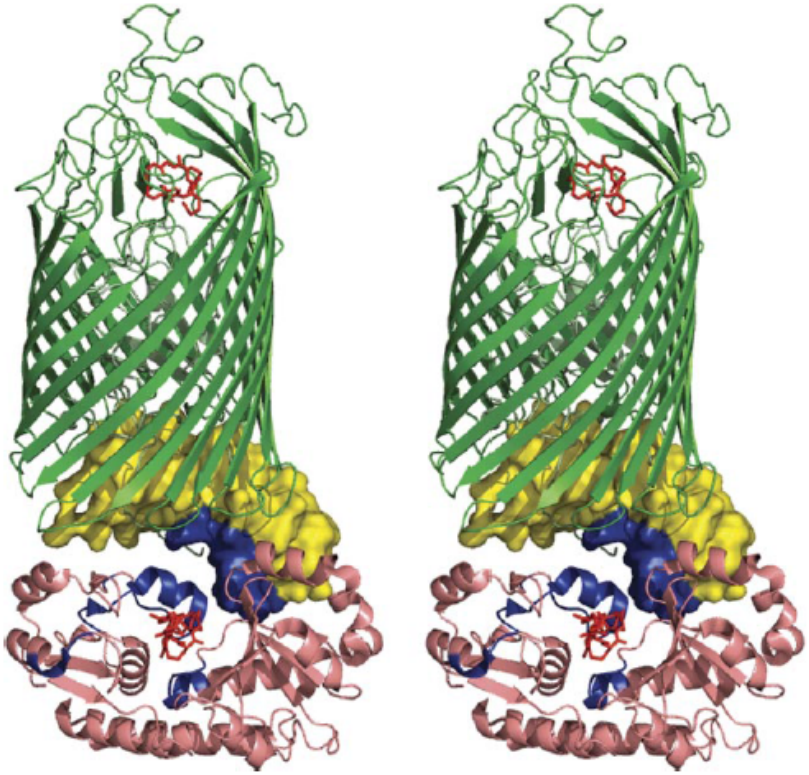


FIGURE 9. **Model of a FhuA-TonB-FhuD ternary complex.** Stereo image depicting a possible ternary complex between FhuA, TonB, and FhuD. FhuD (PDB code 1EFD) was manually docked under the TonB-FhuA crystal structure (PDB code 2GRX) using phage display-identified protein-protein interaction surfaces as docking constraints. Complementary phage display-identified surfaces are colored *blue* on both TonB (*yellow*, surface representation) and FhuD (*salmon*, ribbon representation). The orientation localizes the FhuD siderophore-binding site beneath the lumen of FhuA (*green*, ribbon representation). For clarity, a molecular surface is projected on TonB.