Smallwood et al., http://www.jgp.org/cgi/content/full/jgp.201311159/DC1

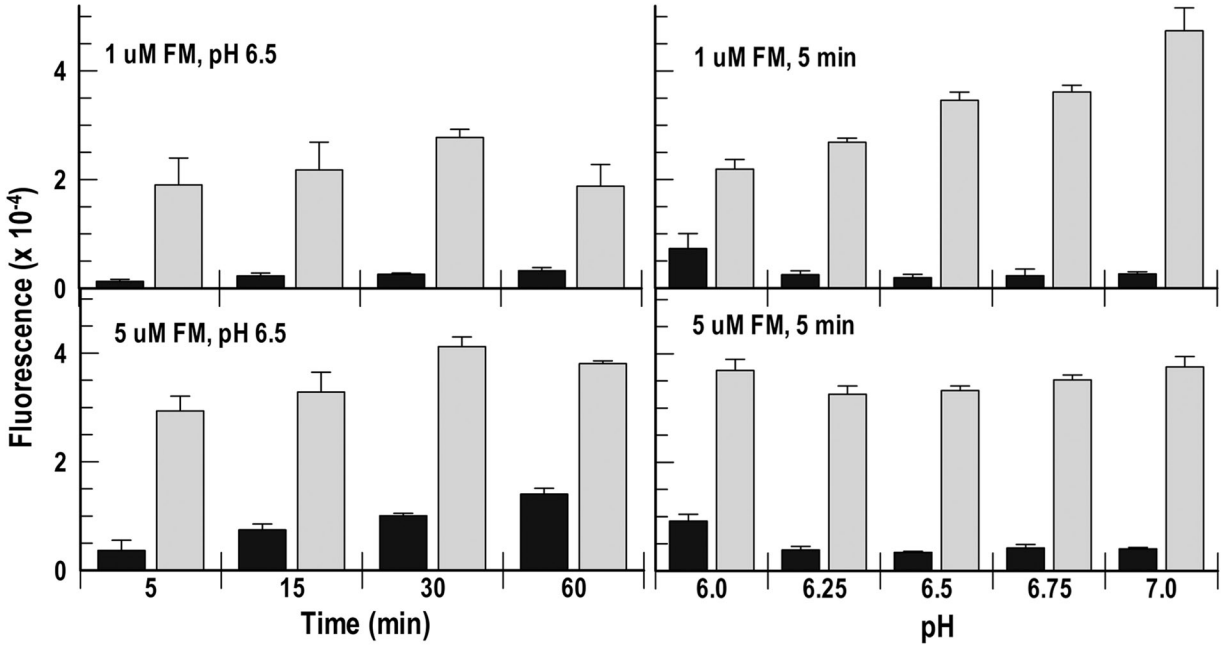

Figure S1. FM labeling reactions. *E. coli* OKN3/p*fepA*$^+$ (black bars) and OKN3/p*fepAS271C* (gray bars) were grown in MOPS media, washed with and resuspended in 50 mM $NaHPO_4$ buffer at the indicated pH values, and incubated with 1 or 5 µM FM for the indicated times. We stopped the labeling reactions with 100 mM BME, washed with PBS, and recorded fluorescence with excitation at 488 nm and emission at 520 nm in an OLIS-SLM8000 fluorometer. Error bars indicate SD of the mean values.

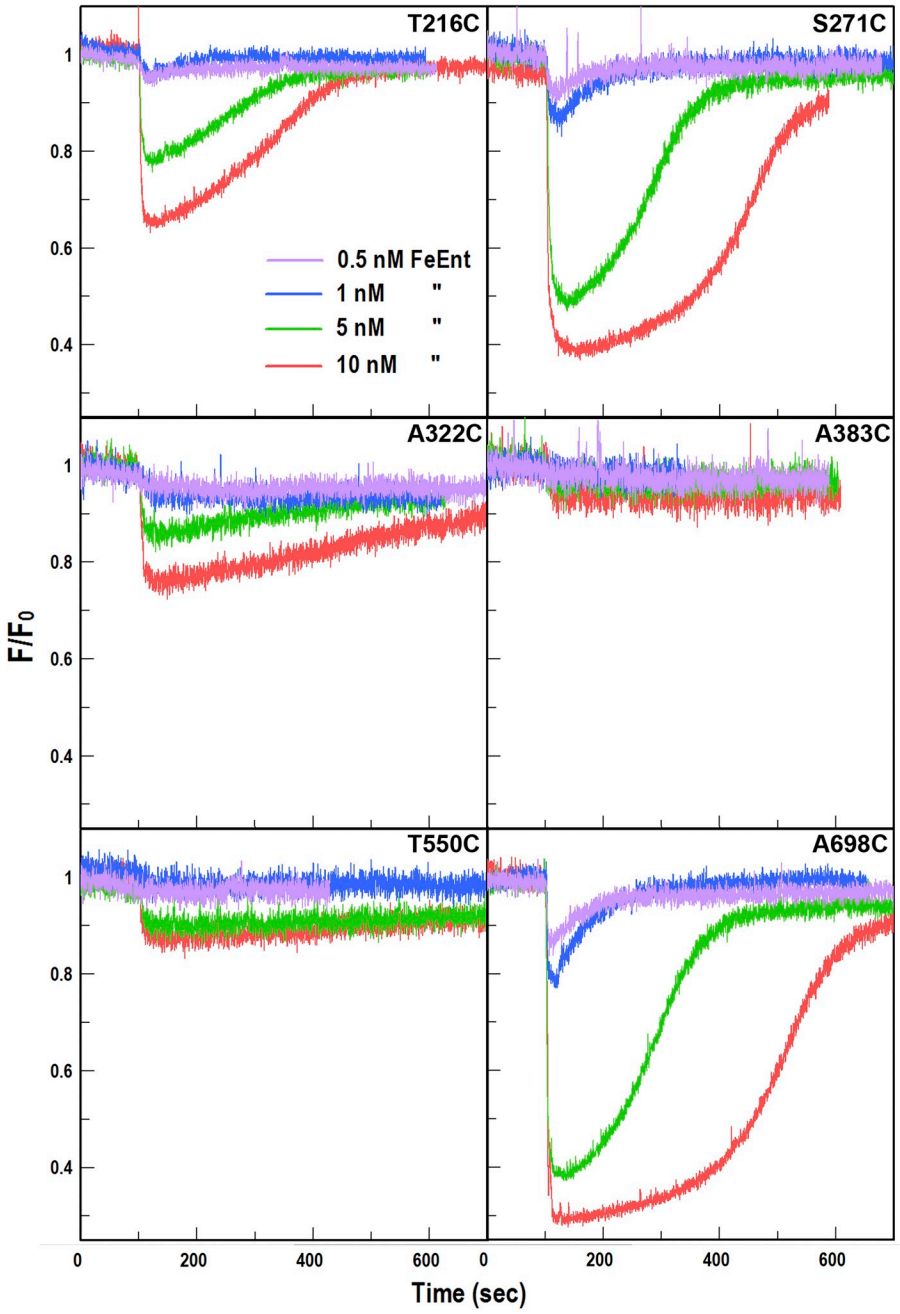

Figure S2. Concentration dependence of FeEnt quenching and recovery. We tested each of the seven FepA Cys substitution derivatives, fluoresceinated in individual loops, for concentration-dependent FeEnt quenching and recovery during FeEnt uptake (Cao et al., 2003). We added varying concentrations of FeEnt to 2.5×10^7 cells/ml in a 3-ml cuvette with stirring at 25°C. In each case, the fluorescence recovery time correlated with the amount of FeEnt: higher concentrations resulted in more extended quenching before recovery. Due to substrate depletion at concentrations ≤1 nM, the extent of quenching was much less and recovery time was very fast. Data were analyzed and plotted in GraFit 6.011.

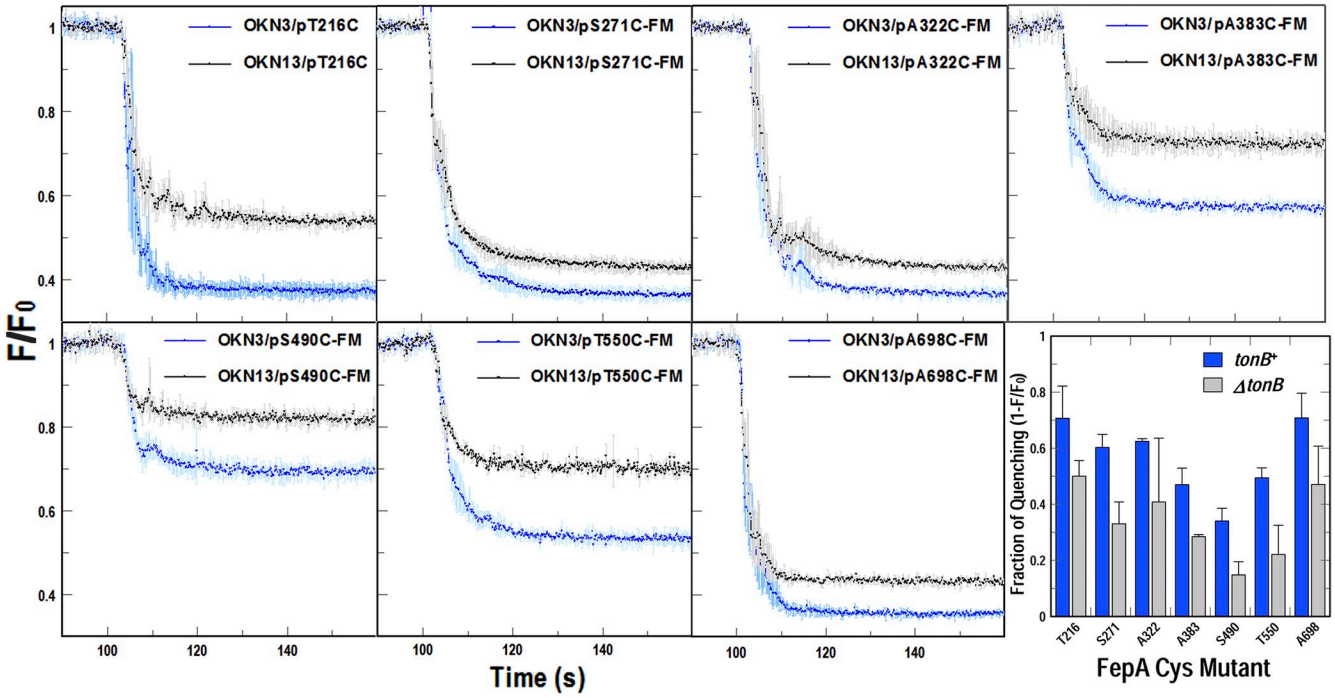

Figure S3. Effect of *ΔtonB* on fluorescence quenching during FeEnt binding by FepA. 2.5×10^7 cells/ml of *E. coli* OKN3 (*tonB*+, *ΔfepA*) or OKN13 (*ΔtonB, ΔfepA*; Ma et al., 2007) expressing FepA Cys substitution mutants labeled with FM (Materials and methods) were observed in a 3-ml quartz cuvette with constant stirring at 4°C. At t = 100 s, we added FeEnt to 100 nM and monitored fluorescence intensity for an additional minute. We performed the experiment three times for each mutant and averaged the curves. The ferric siderophore always quenched fluorescence more in *tonB*+ (blue curves; gray SD error bars) than *ΔtonB* (black curves; cyan SD error bars) bacteria. The bottom right panel summarizes the different mean extents of quenching from two averaged experiments. Error bars on the bottom right indicate SD of the mean values.

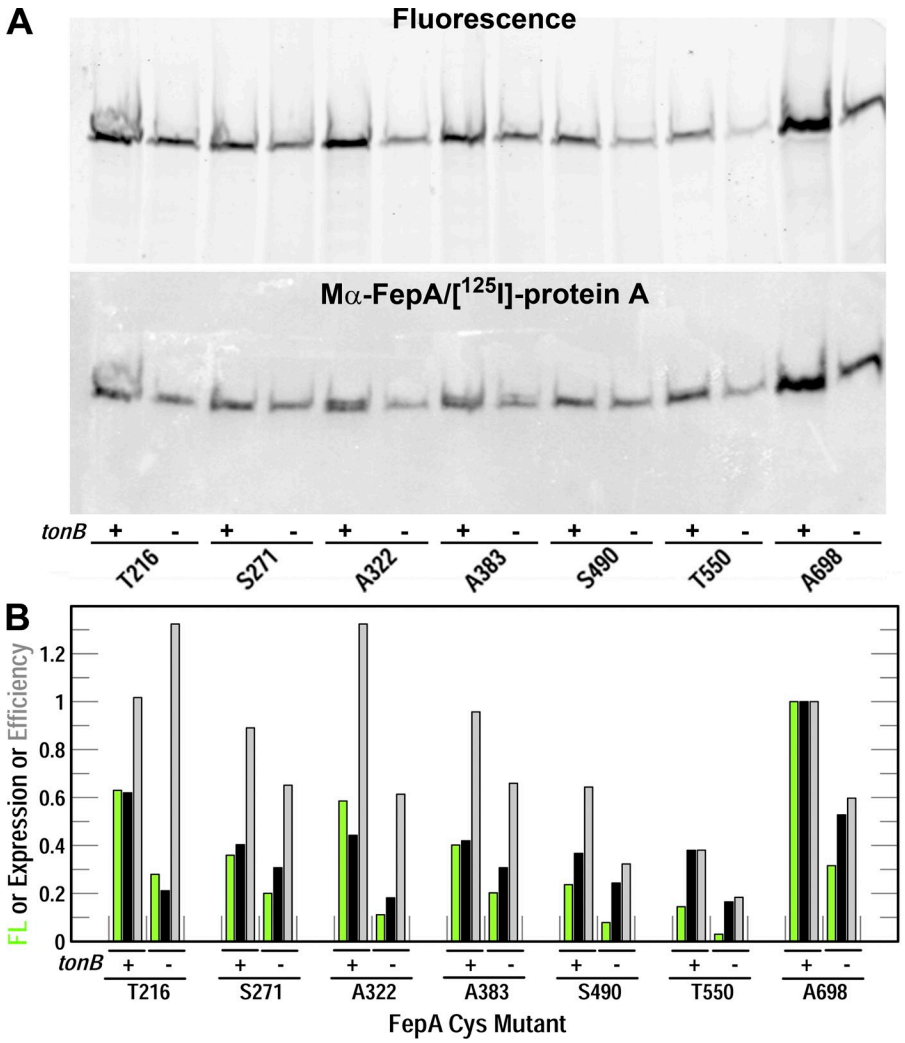

Figure S4. Expression and fluoresceination of FepA in *tonB*$^{+}$ and *ΔtonB* bacteria. (A) We prepared cell lysates from equivalent amounts (5×10^7 cells) of the FM-labeled bacteria analyzed in Fig. S3, solubilized them in SDS-PAGE sample buffer, and resolved equal amounts by electrophoresis. We directly measured the fluorescence (top) of the resolved proteins on a Typhoon imager (GE Healthcare), electrophoretically transferred the proteins from the gel to nitrocellulose, and evaluated FepA expression (bottom) by immunoblots with anti-FepA mAbs 41/45, developed with [^{125}I]protein A. (B) Relative levels of FepA expression, fluoresceination, and labeling efficiency. Green bars show the relative FM labeling level (FL) of each mutant, black bars show the relative expression (E) of each mutant, and gray bars show the relative labeling efficiency (FL/E) for each genetically engineered Cys residue, which significantly varied among the seven loop sites we used. In this experiment we grew all seven mutants on the same day in the same media, labeled them with the same solution of FM, etc., ran a gel, scanned it on the Typhoon imager, transferred the proteins to NC, and immunoblotted with anti-FepA mAb 41/45, followed by [^{125}I]protein A.

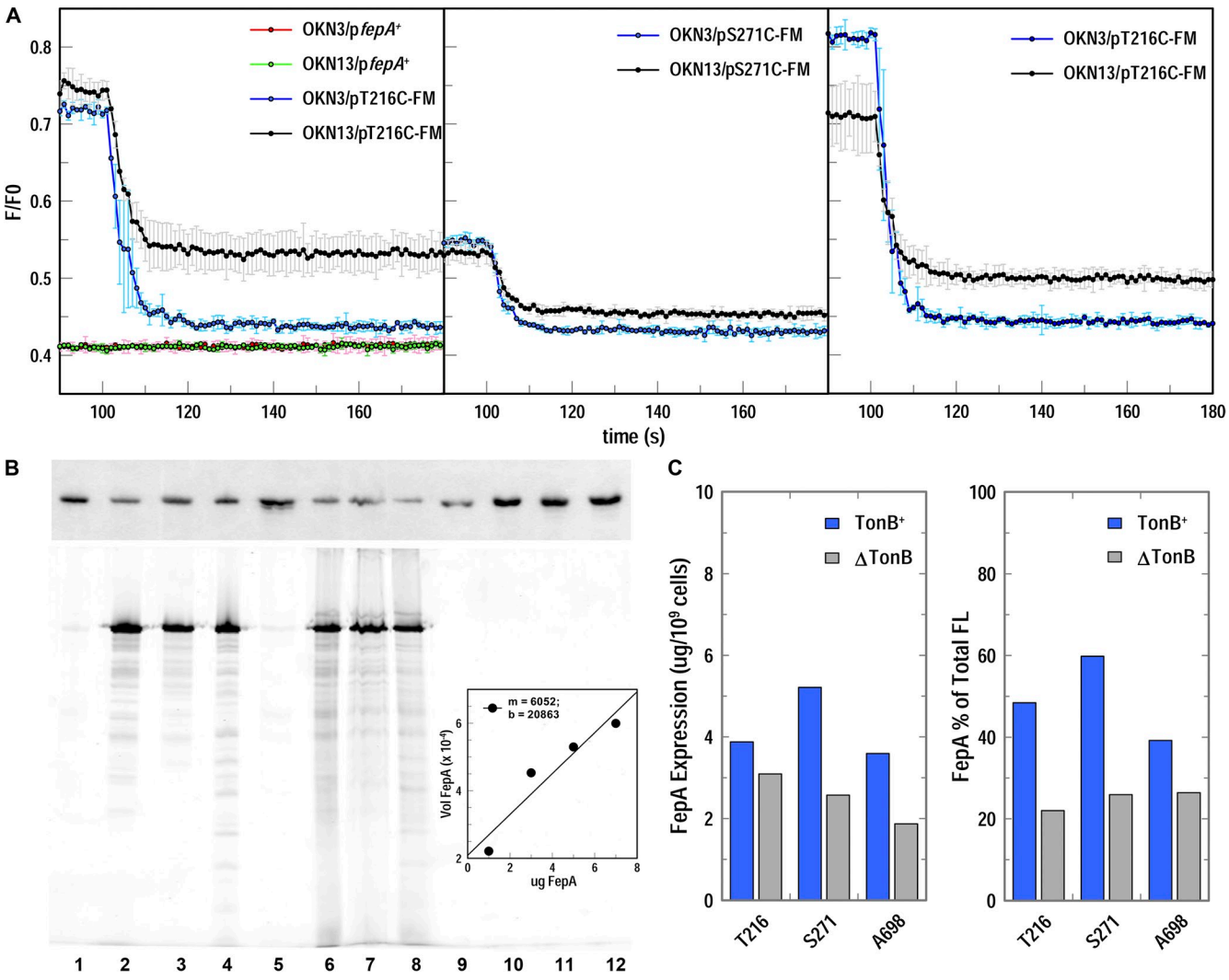

Figure S5. Analysis of FepA Cys mutant fluoresceination in OM fragments. In a separate trial from the experiment in Figs. S3 and S4, we cultured and labeled OKN3 or OKN13 carrying pHSG575 plasmids with *fepA*$^+$ or *fepA* mutants T216C, S271C, or A698C. (A) Live cell fluorescence. We labeled cells with FM as in Fig. 3 and recorded fluorescence at 2.5×10^7 cells/ml in an OLIS-SLM8000 fluorometer. Background fluorescence was identical in OKN3/p*fepA*$^+$ (*tonB*$^+$; green tracing in the left panel) or OKN13/*pfepA*$^+$ (*ΔtonB;* red tracing in the left panel) cells, but each of the three FM-labeled FepA Cys mutants (T216C, left; S271C, center; A698C, right) was quenched more by FeEnt (added to 100 nM at 100 s) in the *tonB*$^+$ host (blue tracings; mean of three trials, with cyan SD error bars) than in the *ΔtonB* strain (black tracings; mean of three trials, gray SD error bars). (B) FepA fluorescence and concentration. (bottom) 10 µg of cell envelope protein (see Materials and methods) from OKN3 (lanes 1–4) or OKN13 (lanes 5–8) expressing FepA$^+$ (1 and 5), FepAT216C (2 and 6), FepAS271C (3 and 7), or FepAA698C (4 and 8) was subjected to SDS-PAGE and fluorescence imaging on a Typhoon scanner. (top) We also measured FepA expression by Western immunoblotting with anti-FepA mAbs 41/45/[^{125}I]protein A and phosphorimagery; lanes 9–12 contained 1, 3, 5, and 7 µg of purified FepA, respectively. The inset shows the linearity of FepA quantification by this method. (C) Relative fluorescence in *tonB*+ and *ΔtonB* strains. The fluorescence and concentration analyses (ImageQuant) of the gel and immunoblot in A and B showed less FepA, and a lower fraction of FepA fluorescence relative to total fluorescence, in the cell envelopes of the *ΔtonB* host strain.

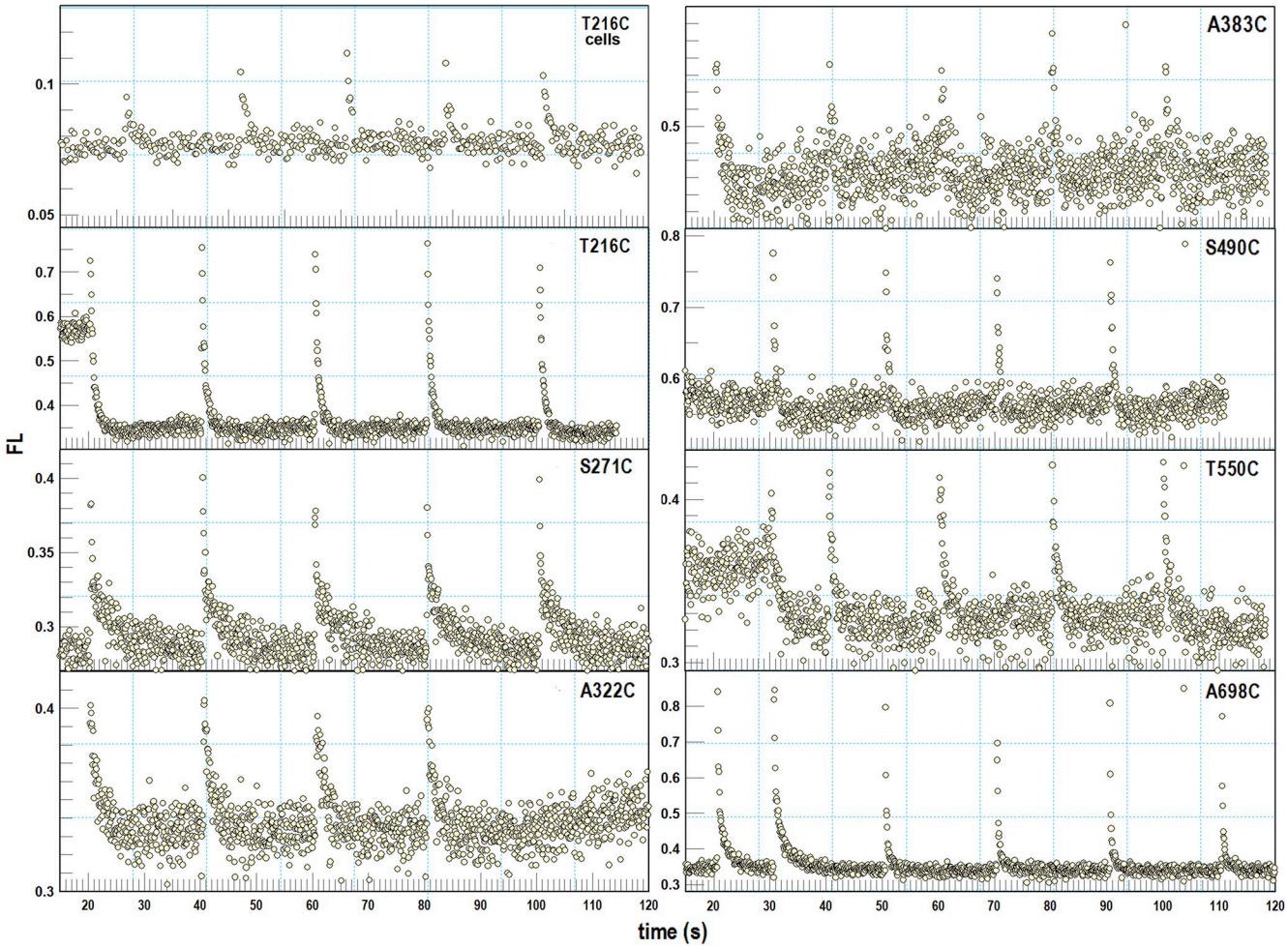

Figure S6. Raw data from rapid-mixing stopped-flow analyses of FeEnt-mediated fluorescence quenching. Stopped-flow experiments were performed as described in Fig. 2. The figure shows quenching profiles from each of the seven FM-labeled surface loops in 50 mM $NaHPO_4$ buffer, pH 7.4. The top left panel shows rapid-mixing stopped-flow data for intact, live bacterial cells expressing FepAT216C-FM; all other panels derive from analysis of fluoresceinated FepA Cys substitution mutants in OM fragments. These data were the basis of the quenching tracings shown in Fig. 2, and the ensuing rate constant calculations in Table 1.

Table S1

Summary of FepA Cys substitution mutant phenotypes

	N domain										C domain								
Location	**TonB-box**		**Periplasm**			**Interior**			**Loops**		**Loops**								**Periplasm**
Residue	**12**	**14**	**30**	**32**	**33**	**54**	**59**	**127**	**63**	**101**	**216**	**271**	**280**	**322**	**383**	**482**	**550**	**698**	**666**
Expression[1]	**100**	100	100	100	100	100	100	60	100	100	100	100	100	100	100	100	100	100	100
Functionality of Cys substitution mutants before fluoresceination																			
Binding[2]	25	100	100	100	100	60	40	25	40	30	47	88	100	59	82	10	48	100	100
Transport[3]	20	40	100	100	100	100	30	0	20	10	77	84	100	62	45	0	28	100	100
Susceptibility to fluoresceination at 0°C																			
5 μM[4]	50	25	52	44	38	63	45	13	25	100	125	100	35	50	75	38	13	100	50
300 μM[5]	75	ND	90	88	60	ND	ND	ND	ND	ND	ND	100	100	ND	ND	ND	ND	100	75
Susceptibility to fluoresceination at 37°C																			
5 μM[4]	30	10	60	52	20	20	15	20	120	90	80	100	45	120	100	65	70	80	20
300 μM[5]	90	ND	100	100	90	ND	ND	ND	ND	ND	ND	100	100	ND	ND	ND	ND	100	100
Functionality of Cys substitution mutants after fluoresceination																			
Binding[2]	17	83	100	90	83	33	40	7	13	13	43	81	100	21	19	10	67	87	90
Transport[3]	7	53	100	100	100	53	27	0	13	0	37	60	100	19	32	0	26	86	100

We transformed *E. coli* OKN3 (*ΔfepA*; Ma et al., 2007) with pHSG575 plasmids carrying *fepA* with site-directed Cys substitutions. We grew cells in MOPS media and labeled them with FM (see Materials and methods) at 5 or 300 μM. Dark grey columns note sites that were well labeled by FM or A_{546}M at 5 μM; light grey cells note sites that were only well labeled by FM at 300 μM, and not modified by A_{546}M. We tested the same cells for ^{59}FeEnt binding capacity and transport velocity before and after modification, analyzed lysates by SDS-PAGE and Western immunoblotting with anti-FepA mAb 41/45/[^{125}I]protein A immunoblots (Ma et al., 2007), and quantified expression with ImageQuant. We repeated each determination at least three times.

[1]Expression. Percent expression relative to wild-type FepA, standardized by gels with purified FepA (see Fig. S4). Mean standard error for expression was 15.7%.

[2]Binding. Percentage of wild-type FepA ^{59}FeEnt binding capacity was determined by filter assays (Newton et al., 1999). We subtracted nonspecific background cpm (OKN3) from the samples to yield the tabulated values. Mean standard errors for capacity before and after fluoresceination were 6.1% and 9%, respectively.

[3]Transport. Percent of wild-type FepA ^{59}FeEnt uptake rate. Mean standard errors for V_{max} before and after fluoresceination were 9.3% and 10.86%, respectively.

[4]Percent of maximum labeling at 5 μM FM (relative to S271C), quantified by image analysis (ImageQuant). Mean standard error was 13.8%.

[5]Percent of maximum labeling at 300 μM FM (relative to S271C), quantified by image analysis (ImageQuant). Mean standard error was 17%.

ND, no data.

TABLE S2

Binding and transport by cells expressing FepA surface loop Cys substitutions

Site	Loop	Binding				Transport				
		<FM		>FM		<FM		>FM		
		K_d	Cap	K_d	Cap	K_M	Vmax	K_M	Vmax	$t_{1/2\ Rrecovery}$ (rank)
fepA+	NA	1.5	110.8	2.2	111.7	1.2	124	2.2	99.7	NA
T216C	2	0.8	27.8	5.22	18.5	2.1	86	2.1	40.6	103 (2)
S271C	3	2.1	97.8	4.0	90.4	1.5	93.5	2.3	67	69 (1)
A322C	4	2.9	65.4	4.3	30.7	1.7	68.8	4.3	21	193 (4)
A383C	5	6.7	91.9	4.7	20.7	3.0	50.4	6.2	35.6	630 (6)
S490C	7	15	64	90	22	8.9	65	57	35	700 (7)
T550C	8	1.7	53.2	3.3	30.7	2.2	125.7	2.9	29.5	315 (5)
A698C	11	1.1	18.5	4.7	36.7	1.9	124.2	2.8	96.3	165 (3)

Affinities and rates were determined by ^{59}FeEnt binding and uptake assays (Newton et al., 1999). We measured binding K_d (nanomolar) and capacity (Cap; pMol/10^9 cells) before and after modification with FM. We performed each determination twice; the mean standard errors for K_d and capacity before fluoresceination were 21% and 10%, respectively, and after fluoresceination they were 22% and 16%, respectively. We also measured transport K_M (nanomolar) and V_{max} (pMol/min/10^9 cells) before and after modification with FM. We performed each determination twice; the mean standard errors for K_M and V_{max} before fluoresceination were 26% and 11%, respectively, and after fluoresceination they were 39% and 19%, respectively. Lastly, we spectroscopically observed the half-time ($t_{1/2}$) of fluorescence recovery after FeEnt quenching as a measure of depletion of the ferric siderophore from solution by cellular transport (parenthetic values rank the seven loops). We performed each determination at least twice; the mean standard errors for the recovery half-times were 11%.

REFERENCES

Cao, Z., P. Warfel, S.M. Newton, and P.E. Klebba. 2003. Spectroscopic observations of ferric enterobactin transport. *J. Biol. Chem.* 278:1022–1028. http://dx.doi.org/10.1074/jbc.M210360200

Ma, L., W. Kaserer, R. Annamalai, D.C. Scott, B. Jin, X. Jiang, Q. Xiao, H. Maymani, L.M. Massis, L.C. Ferreira, et al. 2007. Evidence of ball-and-chain transport of ferric enterobactin through FepA. *J. Biol. Chem.* 282:397–406. http://dx.doi.org/10.1074/jbc.M605333200

Newton, S.M., J.D. Igo, D.C. Scott, and P.E. Klebba. 1999. Effect of loop deletions on the binding and transport of ferric enterobactin by FepA. *Mol. Microbiol.* 32:1153–1165. http://dx.doi.org/10.1046/j.1365-2958.1999.01424.x