Gram-Negative Bacterial Iron Acquisition Systems:
Signal and Energy Transduction Drive Siderophore Uptake through the Cell Envelope

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I. Signal and energy transduction through bacterial membranes. The thin biological membranes between a cell's interior and its environment encompass many indispensable functions. Most fundamentally, they create an inward flow of nutrients to supply metabolic precursors for the biochemical pathways that constitute life. But, transport is a selective phenomenon: not all molecules penetrate into cells, because membranes create a permeability barrier that attunes each cell to its individual environment. This acquisition of desirable substances and rejection of undesirable or noxious compounds is a focal point of our research. We are biochemically characterizing active transport processes that internalize compounds against their natural concentration gradients. Electrochemical potential created by ion gradients across membrane bilayers often powers thermodynamically unfavorable uptake reactions. In these contexts the proteins of bacterial cell membranes sense the environment, identify metabolically beneficial nutrilites, and capture them by membrane transport that involves signal and energy transduction by multicomponent protein assemblies. We are researching one such complex of proteins (TonB-dependent iron transport systems) in Gram-negative cells, and another network of cell envelope proteins (sortase-dependent and independent heme/hemoglobin transporters) in Gram-positive cells. Both systems are potential targets for antibiotic discovery, which is one of our ultimate goals, and both present opportunities for the application of novel technologies to understand biochemical mechanisms. Relevant to the latter end, we are employing site-directed extrinsic fluorescence spectroscopic approaches to determine molecular environments and conformational motion within target membrane proteins during performance of their biological function. These derive from measurements of quenching and resonance energy transfer (FRET), as well as protein anisotropy and lateral motion in membrane bilayers using single molecule spectroscopy and total internal reflection fluorescence microscopy (TIRFM).

II. Bacterial iron uptake systems. We are studying the biochemical mechanisms that prokaryotes use to obtain $\mathbf{Fe^{+++}}$ from their environments, and the function of these systems in the pathogenesis of human and animal hosts. The passage of metal ions through membranes raises questions about its mechanisms, energetics, and the thermodynamics/kinetics of the transport events (8, 9, 10). We are characterizing iron uptake in two structurally distinct systems: the Gram-negative bacterial outer membrane (OM), and the Gram-positive bacterial cytoplasmic membrane (CM). Both systems function by active transport. The energetics underlying OM metal transport are as yet uncertain, but CM iron permeases are usually ABC-transporters, driven by ATP hydrolysis. Research on the relationship of iron metabolism to bacterial pathogenesis is summarized by a few statements. Iron is a valuable commodity in metabolism, and therefore a key element of bacterial pathogenesis. Not just microorganisms, but virtually all organisms require iron for cellular processes, including electron transport and energy generation by heme-containing proteins, DNA synthesis, catabolism, and detoxification of reactive oxygen species. Humans and animals sequester iron in proteins like transferrin, lactoferrin and ferritin, in large part as a means of defense against microbial infections. Microorganisms, on the other hand, synthesize and secrete small molecules called siderophores (*Gr. "iron-carrier"*), that chelate iron and actively remove it from eukaryotic binding proteins. Finally, some pathogens survive in the host by directly removing iron from transferrin, lactoferrin and ferritin, without elaborating siderophores.

A. Site-directed spectroscopy. One object of our investigations is a mechanistic understanding of membrane transport events. Toward this end we utilize molecular biology to introduce biophysical probes at sites of interest in transport proteins, and then observe biochemical activity during ligand uptake (5, 6, 12, 14, 15). We accomplish this aim in two stages: site-directed substitution mutagenesis to insert single cysteines in a target protein, followed by maleimide-, iodoacetamide- or thiosulfonate-mediated modification of the sulfhydryl side chain with paramagnetic or fluorescent reagents. The environmental sensitivity of such probes (Fig. 1) often allows direct observation of conformational dynamics as a transporter internalizes its substrates. Our initial experiments with these techniques focused on FepA, the ferric enterobactin (FeEnt) receptor of Gram-negative bacteria. FepA resides in the Gram-negative bacterial OM and transports the iron complex of the siderophore enterobactin (FeEnt). The *E. coli* system typifies those of *Salmonella typhi*, *Vibrio cholerae*, *Shigella dysenteria*, *Neisseria meningitidis*, *Bordetella pertussis* and *Yersinia pestis*.

Our findings on FepA first showed the presence of a large transmembrane, porin channel in its interior (13, 20), and then found unexpectedly dynamic conformational motion during uptake of ligands through the pore (5, 6, 14, 22) We were not the first to utilize site-directed spectroscopic techniques (1) to analyze membrane proteins, but we were the first to apply these methods in living cells to detect the sub-reactions of a transport cycle through the observation of

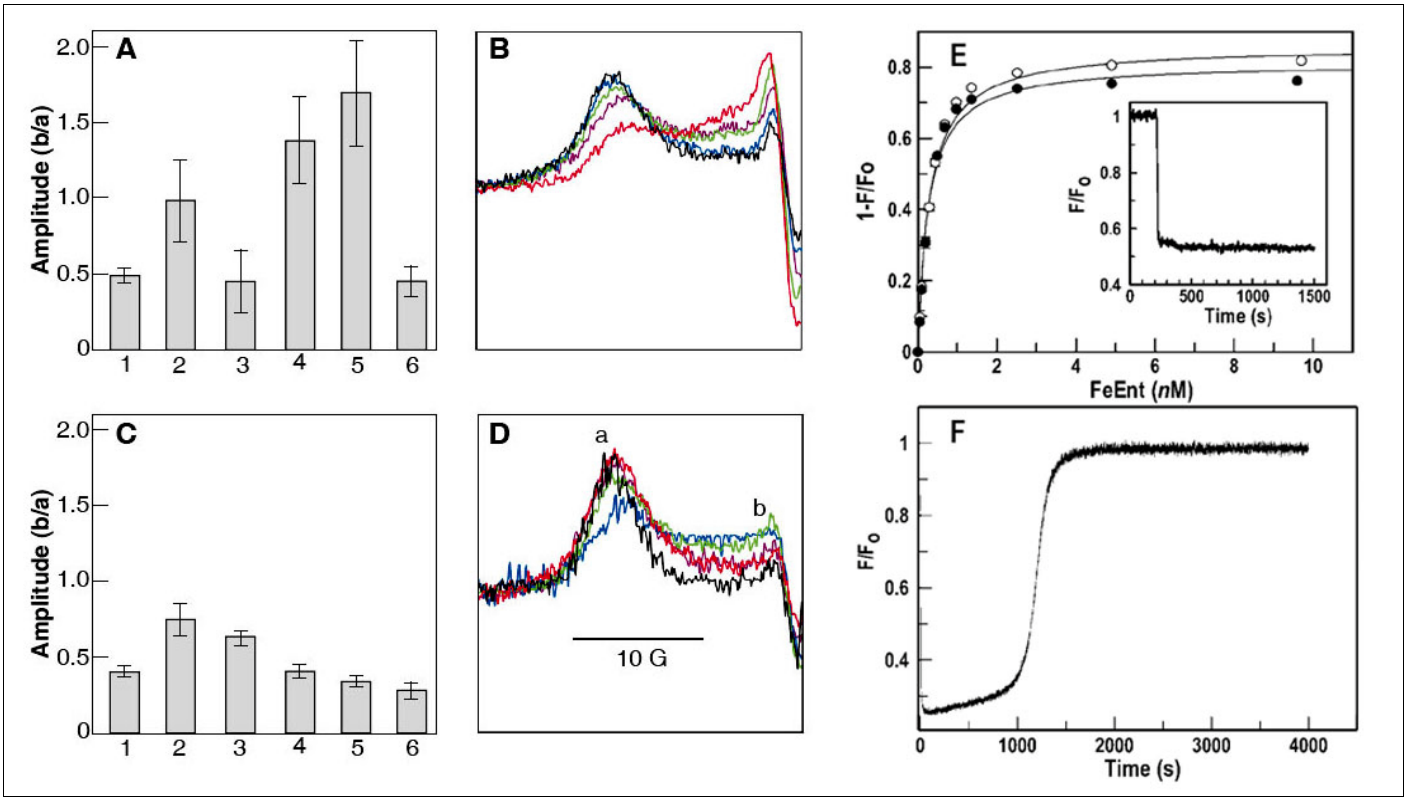

Figure 1. Spectroscopic measurements of active transport through FepA. (A-D) Electron Spin Resonance measurements of FeEnt and colB uptake (6). (A and C) FeEnt– and colB–induced variations in spin labeled FepA motion. *E. coli* cells were grown, spin-labeled, and exposed to ligands in the EPR spectrometer. Multiple signal averaged spectra were collected from independent preparations of bacteria, and the mean and standard deviation of the ratio of weakly and strongly immobilized probes (b/a; see panel D) was calculated after the addition of FeEnt (A) (n = 4) and colB (C) (n = 6). The effects of the siderophore and colicin were also studied by other methods, including the absolute amplitudes, areas, separation of low- and high-field extrema (2T‖') and half-width at half-height for peaks a and b. All these showed the tendency of the siderophore to mobilize spin labels and the colicin to immobilize them. Bars 1 through 6 are from scans at sequential times (~3 min intervals) after ligand addition ligands . (B and D) Superimposed spectra from single experiments are shown in the region of the strongly and weakly immobilized peaks. (B) Spectra were collected in the absence of FeEnt at 37°C (purple), and in its presence at 4°C (blue), after warming the sample to 37°C for 5 (green) and 15 (red) min, and after recooling it to 4°C (black). (D) Spectra were collected in the presence of colB at 4°C (purple), after increasing the temperature to 37°C for 5 (blue), 15 (green), and 75 (red) min, and after recooling to 4°C (black). The ESR measurements show time-dependent motion in FepA, that describes its interaction with its two ligands. For FeEnt the data show conformational changes during its passage through FepA. For further details see reference 6. (E and F) Fluorescence spectroscopic measurements of FeEnt binding and transport (5). (E) Binding. When labeled with fluorescein maleimide (FM) at FepA residues S271C or S397C, live *E. coli* show FeEnt binding as quenching of fluorescence intensity (inset). When measured in the presence of varying amounts of FeEnt, the binding isotherm (1-F/F_0 fitted against [FeEnt] using a "Bound versus Total" equation) produced K_d values for FepAS271C-FM and FepAS397C-FM of 0.263 nM and 0.292 nM, respectively, which are the same as K_d values from radioisotopic measurements (18). (F) Transport. The time course of fluorescence was observed in FM-labeled bacteria at 20 °C. FeEnt was added to 10 nM at t = 0, and intensity was measured in the fluorometer. The tracing shows time-dependent conformation motion during the ligand transport reaction. For details see ref. 5.

conformational changes in the transporter itself (5, 6, 15) ***This novel technology enables us to examine dynamic motion in membrane proteins during their physiological activities, potentially superceding the "snapshots" of mechanisms that crystallography provides. Our approach and goal is to use time-resolved fluoresence intensity determinations, polarization studies, FRET, and measurements of susceptibility to external quenchers to characterize membrane transport in living cells.***

B. The OM iron transporters of Enterobacteriaceae: FepA and its relatives. The asymmetric OM bilayer defines the transport properties of Gram-negative bacteria: it permits entry of nutrients and vitamins, but excludes toxic molecules like detergents and certain antibiotics (7-10). OM proteins called porins are the basis of this selectivity, and iron transporters (like FepA) form an unusual subclass of the porin superfamily: they catalyze TonB- and energy-dependent uptake of metals (for FepA, FeEnt and its analogs (8, 9, 10), protein toxins (colicins B and D; (3)), and a virus (bacteriophage H8; (19). Unlike archaetypal porins FepA does not contain an open channel through the OM. Although

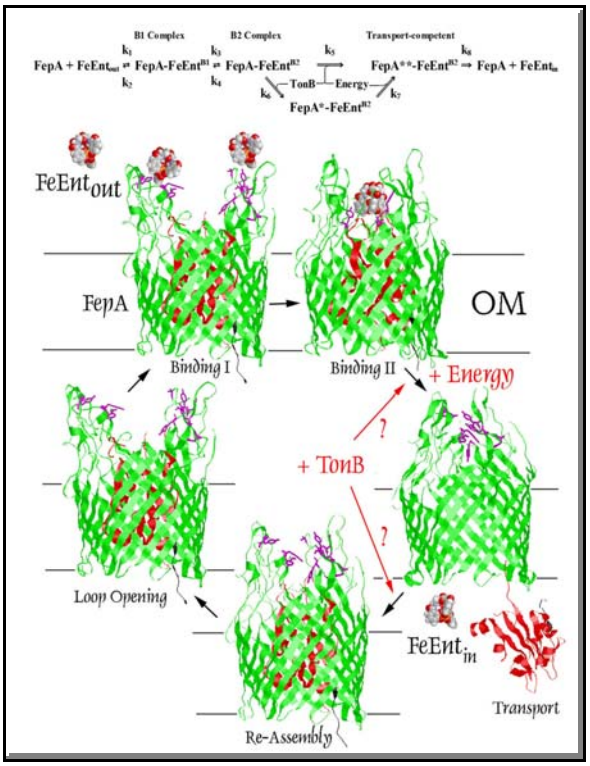

Figure 2. FeEnt uptake by FepA. The N-terminal 150 residues of FepA fold into a globular domain (red) that inserts into a C-terminal 575 amino acid transmembrane β-barrel (green). Initially, the surface loops of the transporter assume an open conformation that is receptive to the binding of ligands. FeEnt associates with aromatic and basic residues in the surface loops, converting the loops to a closed conformation that holds the iron complex above the N-domain, ready for transport. One possible mechanism of transport, the Ball-and-Chain theory, involves dislodgement of the N-domain from the pore, which pulls FeEnt through the membrane bilayer into the cell. Transport requires the input of energy and the actions of TonB, by currently unknown mechanisms.

its C-terminus folds into a β-barrel that makes a channel, its N-terminus forms a globular domain that resides within the lumen of the channel and completely closes it. FepA transports FeEnt in stages. The iron complex initially binds to external loops of the OM protein, and through a series of incompletely understood reactions that involve energy and other proteins (most notably, TonB), the receptor transports the ligand (2, 4, 5, 12, 14, 15, 16, 17, 18, 20, 21) We are researching the physical mechanism of this transport reaction: the 150-residue N-terminus of FepA must change, either by rearranging *in situ* to form an opening through which the ligand passes, or by dislodging from the β-barrel and thereby opening it for ligand passage. Besides the use of biophysical techniques *in vivo*, noted above, we are pursuing biochemical and molecular biological approaches to elucidate aspects of the transport reaction *in vitro*, especially the disposition and potential motion of the N-domain (15).

During uptake of its three classes of biological ligands (iron complexes, protein toxins and bacteriophage) FepA acts in concert with several other cell-envelope proteins, including TonB. The mechanistic details of these interactions are incompletely understood, but TonB is required for all OM metal transport systems (10). When metal complexes or other molecules bind to the surfaces of proteins like FepA (2, 4, 23) they compel conformational changes in the receptors that begin in the outer loop regions and propagate through their transmembrane domains, until they reach the internal surface of the OM bilayer (2, 4, 5, 16, 21, 25). Thus, ligand adsorption results in conformational signal transduction through the membrane, and these structural changes expose protein domains at the N-terminus of the receptor protein that are recognized and bound by the TonB C-terminus. This conjunction of ligand recognition and ultimate uptake through the OM bilayer is an excellent example of membrane signal transduction: the bacterium senses the external environment by the binding affinities of its surface proteins, identifies a desired nutrilite, and then uses protein conformational motion to activate the transport system and capture it. The uptake activities require energy because FepA accumulates FeEnt against a concentration gradient. Nevertheless, active transport is confounding in a bilayer that contains open (porin) channels, because they preclude the formation of an electrochemical gradient. These considerations may explain the need for TonB in OM metal transport. TonB may link OM transporters to the energized inner membrane, and promote the opening of their closed channels by direct physical contact (8 - 10). We found homology in TonB to periplasmic proteins that associate with peptidoglycan (PG), and demonstrated its binding to purified *E. coli* PG (24). The data suggested a membrane surveillance model, in which TonB spans the periplasm to survey the underside of the OM bilayer, locating ligand-bound receptor proteins and facilitating their transport reactions (Fig. 3). ***With fluorescence spectroscopic, biochemical and molecular biological approaches, we are studying the interaction of TonB with other cell envelope proteins (e.g., FepA) and with PG. This research will biochemically define the activities and role of TonB (and accessory proteins) in the facilitation of high affinity metal uptake through the bacterial OM. These phenomena present an opportunity to elucidate transmembrane signal transduction, the bioenergetics of active transport, and a unique, multi-component metal internalization process, all in the same system.***

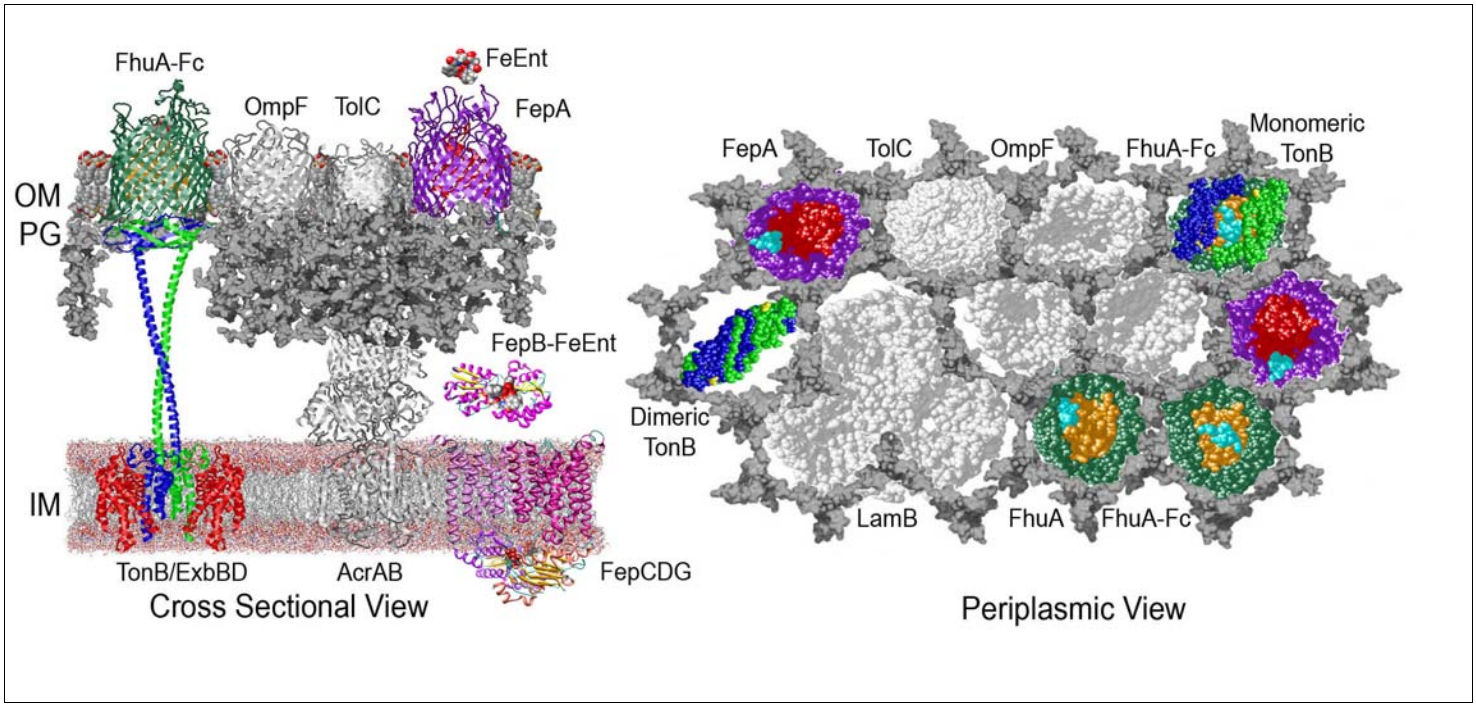

Figure 3. Prototypic TonB- and energy-dependent iron transport systems of the Gram-negative bacterial cell envelope in the "Membrane Surveillance" model of TonB action. The FhuA (green) and FepA (purple) OM transporters require the activity of TonB (blue and light green)/ExbBD (red) and the input of cellular energy to accomplish iron uptake into the periplasm. Once inside, periplasmic binding proteins (e.g., FepB, pink) adsorb the ferric siderophores and transfer them to ABC transporter permeases in the IM (e.g., FepCDG, shades of magenta). The OM transport stage is postulated to involve a physical interaction with the TonB C-terminus. (Left) In a cross-sectional view of the cell envelope, dimeric TonB (green and blue), associates with the IM by α-helices in its N-terminus that complex with ExbBD (red). TonB also contains lengths of rigid polypeptide (shown here as coiled helices) that span the periplasm, and LysM motifs in its C-terminus that associate with the PG layer (grey) underlying the OM bilayer. The TonB dimer has general affinity for PG and TonB-independent OM proteins (e.g., OmpA), which tends to localize the C-terminus at the periplasmic interface of the OM bilayer. The monomeric form of the C-terminus, on the other hand, has a specific affinity for accessible TonB boxes of (ligand-bound) TonB-dependent receptors, resulting in their recruitment by its β-sheet. These affinities allow TonB to physically survey the periplasmic surface of the OM bilayer until it encounters bound LGP (FhuA, dark green and orange). This motion across the internal surface of the OM may derive from movement of the N-terminus in the fluid IM bilayer. FepA (purple and red) and the TonB-independent OM proteins OmpF (white) and TolC (white) are also shown associated with PG. The latter protein complexes with AcrAB in the IM bilayer, which provides a reference for the distance between the IM and OM bilayers. (Right) From a periplasmic view, the dimeric form of TonB (green and blue) moves among the roughly hexagonal, 50 Å cells of the PG polymer (grey), which associate with the β-barrels of OM proteins (TolC, OmpF and LamB, all shown in white). LGP in the OM bilayer may be ligand-free (FepA: purple β-barrel and red N-domain, cyan TonB-box; FhuA: dark green β-barrel and orange N-domain, cyan TonB-box) or ligand-bound (note FhuA at top and bottom right, with TonB-box relocated to the center of the channel). The TonB-C-domain remains dimeric until it encounters a ligand-bound receptor (FhuA-Fc, top right), and then dissociates into a monomeric form that recruits the TonB-box region into its β-sheet. This binding reaction, and additional unknown energy-dependent reactions, promote the unfolding of the LGP N-domain from the barrel and concomitant internalization of its bound metal complex. Crystallographic coordinates for the following proteins were from the RCSB Protein Data Bank (http://pdbbeta.rcsb.org/pdb/home/home.do): FepA (1FEP), FhuA (1BY3, 1BY5), OmpF (1BT9), LamB (1MAL), TolC (1EK9), TonB C-terminus (1IHR, 1QXX). The N-terminus and central regions of TonB, and ExbBD were drawn (no structural data yet exists for these polypeptides), and the PG polymer was drawn from Meroueh et al., 2006. .

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The trilaminar Gram-negative bacterial cell envelope consists of an outer membrane (OM), and inner membrane (IM), and the fluidic space between them, called the periplasm. The three compartments contain macromolecules that determine what can or will enter the cell, in the form of complex transport systems for sugars, nucleotides, amino acids, vitamins and metals. The OM is a crucial element in this process of obtaining the nutrients needed for survival, especially because it also protects bacterial species like *Escherichia, Shigella, Salmonella, Vibrio, Neisseria* and *Yersinia* from noxious agents in their environments, by conferring selective permeability. That is, its biochemical components structurally intertwine to create a barrier with selective

portals that permit passage of small hydrophilic molecules (<600 D), but simultaneously exclude large or hydrophobic molecules. Proteins called "porins" form these uptake portals. They allow general [OM proteins F and C (OmpF, OmpC)] or facilitated (LamB, Tsx) diffusion of solutes from the environment into the periplasm. However, the uptake of iron creates a problem for bacterial cells. The metal is largely insoluble in water or biological solutions, so microbes secrete small organic compounds (siderophores) that solubilize Fe^{+++} by chelating it. This complexation by siderophores (which are called ferric siderophores when they bind iron) initiates the activity of iron in biological systems. But because ferric siderophore molecules are usually larger than 600 D and therefore cannot pass through the OM channels of general or specific porins, Gram-negative bacteria produce another type of OM protein, ferric siderophore receptors, that specifically recognize individual ferric siderophores, bind them with high affinity, and transport them into the periplasm. These OM iron transporters have many of the same structural features as porins, so they constitute a special class called "ligand-gated" porins (LGP). Nevertheless, LGP do not function by diffusion: they accomplish active transport across the OM by accumulating iron against its concentration gradient. This biochemical uptake reaction requires energy and the participation of another cell envelope protein called TonB, so the ferric siderophore receptors are also referred to as TonB-dependent transporters (TBDT)