

Ligand-Specific Opening of a Gated-Porin Channel in the Outer Membrane of Living Bacteria

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Ligand-gated membrane channels selectively facilitate the entry of iron into prokaryotic cells. The essential role of iron in metabolism makes its acquisition a determinant of bacterial pathogenesis and a target for therapeutic strategies. In Gram-negative bacteria, TonB-dependent outer membrane proteins form energized, gated pores that bind iron chelates (siderophores) and internalize them. The time-resolved operation of the *Escherichia coli* ferric enterobactin receptor FepA was observed in vivo with electron spin resonance spectroscopy by monitoring the mobility of covalently bound nitroxide spin labels. A ligand-binding surface loop of FepA, which normally closes its transmembrane channel, exhibited energy-dependent structural changes during iron and toxin (colicin) transport. These changes were not merely associated with ligand binding, but occurred during ligand uptake through the outer membrane bilayer. The results demonstrate by a physical method that gated-porin channels open and close during membrane transport in vivo.

Transport through biological membranes is selective and directional, providing a regulated influx of solutes required for metabolism and an efflux of molecules that function outside the cell. Ligand-gated membrane channels—which exist in a variety of organisms and tissues, from bacteria to the human brain—form specific uptake pathways by binding solutes and moving them through transmembrane pores (1). In the outer membrane [OM (2)] of Gram-negative bacteria, TonB-dependent gated porins transport iron in the form of ferric siderophores (3). These metal chelates are too big (750 daltons) to traverse the OM through the open channels of general porins (4), and channels large enough to accommodate them pose a threat to bacteria, because they would also allow the entry of detergents, antibiotics, and other noxious molecules. Gated porins have evolved that solve this nutritional dilemma by elaborating ligand-binding surface loops that close their large channels. The acquisition of iron through ligand-gated porins is a determinant of bacterial pathogenesis (5), and the pores themselves are routes for the entry of tox-

ins (6) and antibiotics (7). A prototypical gated porin, *Escherichia coli* FepA, binds the siderophore ferric enterobactin in closed surface loops (8) and transports it through an underlying transmembrane channel (9) into the cell. The latter internalization reaction requires the input of energy and the participation of another protein, TonB (10). Because the TonB NH₂-terminus resides in the inner mem-

brane (11) and TonB promotes uptake through channels in the outer membrane, TonB-mediated transport reactions involve energy transduction between two distinct bilayers.

The outer leaflet of the OM contacts the external environment, and various nutrients, noxious agents, and small molecules interact with OM proteins during the initial stages of transport (12). Because ferric enterobactin binds to the outside of a closed channel, through which it subsequently passes, conformational changes in FepA surface loops are a fundamental part of its suspected transport mechanism. To investigate this possibility, we reacted nitroxide spin labels with a genetically engineered cysteine residue in a ligand-binding surface loop [PL5 (13)] and analyzed the live bacteria by electron spin resonance (ESR) spectroscopy during transport. ESR detects the presence of unpaired electrons, and the introduction of a stable nitroxide free radical creates a molecular probe that conveys information about the structure and motional dynamics at its site of attachment. Site-directed spin labeling has generated extensive information about membrane proteins in vitro (14), including determinations of secondary and tertiary structure and characterization of conformational change. Previous ESR results with the purified mutant protein Glu²⁸⁰ → Cys²⁸⁰ (E280C) (15), labeled with either S-(*o*-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methane

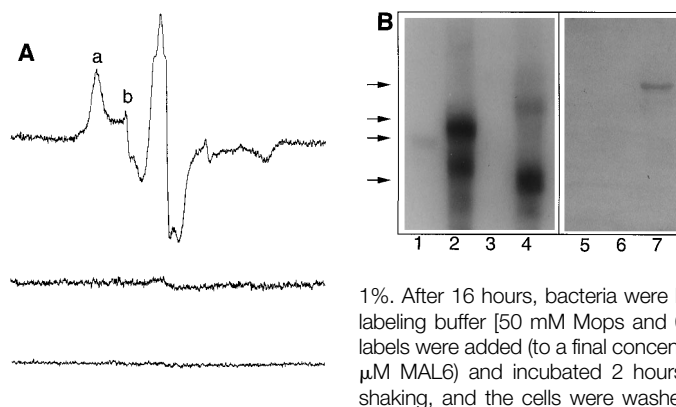


Fig. 1. Nitroxide labeling of FepAE280C in vivo. **(A)** ESR analyses of MTSL-labeled bacteria expressing FepAE280C. KDF541 (9) containing pITS449 or pFepAE280C was grown in LB medium and subcultured into T medium plus appropriate nutritional supplements at

1%. After 16 hours, bacteria were harvested and suspended in labeling buffer [50 mM Mops and 60 mM NaCl (pH 7.5)]. Spin labels were added (to a final concentration of 20 μ M MTSL or 10 μ M MAL6) and incubated 2 hours at room temperature with shaking, and the cells were washed twice with reaction buffer

containing 0.05% Tween-20 and once with reaction buffer alone. After the labeling reaction, the viability of the bacteria was confirmed by plating on LB agar. For ESR analysis, 10¹⁰ cells were suspended in 100 μ l of 0.01 M NaHPO₄ (pH 6.9) containing 0.4% glucose. X-band spectra of MTSL-labeled KDF541/pFepAE280C (top), KDF541/pITS449 (*fepA*⁺; center), or KDF541 (*fepA*; bottom) were recorded on a Bruker EMX spectrometer, operating at 9.8 GHz with a total scan range of 150 G and 100-kHz modulation frequency, at 4°C in a quartz flat cell. Peaks a and b show the position of strongly and weakly immobilized spin labels, respectively. **(B)** [¹²⁵I]-labeled protein A Western blots with anti-MAL6 sera. MAL6 was coupled to bovine serum albumin (BSA) and ovalbumin (OVA). New Zealand White rabbits were immunized with BSA-MAL6 conjugates [100 μ g/week for 1 month (29)], and polyclonal serum was collected in week 5 and assayed for antigen specificity by enzyme-linked immunosorbent assay (30) and Western blots against BSA, BSA-MAL6, OVA, and OVA-MAL6 (lanes 1 through 4, respectively). Lysates (5 \times 10⁷ cells) of KDF541, KDF541/pITS449, and KDF541/pFepAE280C, previously labeled with MAL6, as described above, were reacted with the anti-MAL6 polyclonal serum (lanes 5 through 7, respectively). Arrows indicate the positions of (top to bottom) FepAE280C-MAL6, BSA-MAL6, BSA, and OVA-MAL6.

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thiosulfonate (MTSL) or 4-maleimidotempo [MAL6 (16)] and studied in detergent or liposomes (17), suggested that FepA could be specifically modified and analyzed in vivo (18). Two findings confirmed this expectation: Bacteria harboring *pFepAE280C* were labeled 30- to 50-fold more than the *fepA* host strain KDF541 or KDF541 expressing the *fepA*⁺ allele that lacks the E280C mutation (Fig. 1). Furthermore, in Western blots (protein immunoblots) of cell lysates with anti-MAL6 sera, the engineered Cys residue at FepA residue 280 was the major spin-labeled site in the bacteria (Fig. 1).

Although the in vivo ESR spectra of MTSL attached to residue 280 closely resembled its in vitro spectra (17), marked changes appeared during FepA-mediated transport in live bacteria. MTSL-labeled FepA showed a two-component spectrum in vitro, with the majority of its spins strongly immobilized and a small percentage (<1%) weakly immobilized (17, 19). FepAE280C-MTSL showed similar spectra in vivo at 4°C [3% weakly immobilized (peak b in Fig. 2A)], but an increase in sample temperature to 37°C in the presence of the siderophore shifted a significant proportion of the spin-label population [16% in Fig. 2E; (20)] to the weakly immobilized state (Fig. 2). The effects of ferric enterobactin on FepA in vivo were distinct from those previously observed in vitro, and the binding of the siderophore caused a slight further immobilization of MTSL at E280C (17). The mobilization of spins in vivo was so conspicuous that we initially suspected chemical release of MTSL from FepA by ferric enterobactin. Other experiments refuted this possibility though. Recooling of the sample to 4°C reversed the effects, regenerating the properties and proportion of strongly immobilized spins attached to E280C (Fig. 2). Secondly, MAL6, which forms a chemically stable thioether bond with Cys, produced the same results with FepAE280C in vivo (21). Double integration of the X-band spectra, furthermore, showed that spin labels were neither destroyed nor lost during the experiment (Fig. 2). The conversion of spins from strong to weak immobilization was comparable to changes in MTSL motion that accompany FepA denaturation (22), strongly suggesting that the siderophore stimulates relocation of PL5 away from globular protein structure into the aqueous milieu. These data show that two distinct conformations of PL5 exist in vivo: a form that binds the siderophore, in which spin-label motion is restricted, and a form that arises after siderophore binding, in which spin-label motion is comparatively unrestricted. The ap-

pearance of the mobilized form coincided with the initiation of siderophore transport (23), suggesting that ferric enterobactin triggered the conformational changes

in FepA, either before or during its uptake.

Another TonB-dependent ligand that penetrates via FepA, colicin B, also altered MTSL mobility during its transport, but the effects were opposite to those engendered by the siderophore. The addition of saturating colicin B at 37°C progres-

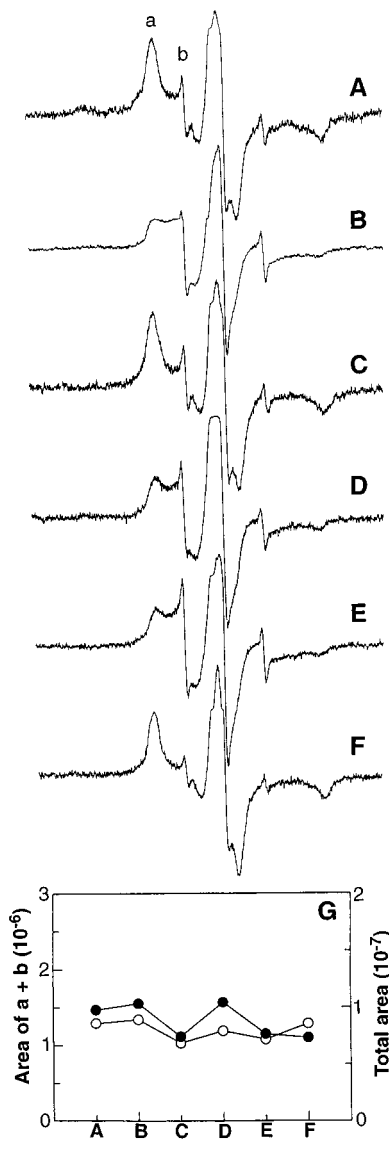


Fig. 2. Effect of ferric enterobactin on the mobility of MTSL attached to FepAE280C in live bacteria, observed in signal-averaged X-band spectra. KDF541/*pFepAE280C* was grown, spin-labeled, and prepared as described in Fig. 1. Signal-averaged X-band spectra (six field sweeps collected over an 8-min period) of spin-labeled bacteria were recorded at 4°C (A) and again after warming the flat cell to 37°C [B (31)]. A fresh aliquot of cells from the same labeling reaction was suspended in saturating ferric enterobactin (300 μM) at 4°C (C), the sample was warmed to 37°C, and spectra were recorded after 5 (D) and 15 (E) min (midpoints of the signal-average measurements). The bacteria were then cooled again to 4°C for 30 min, and spectra were recorded (F). Each of the spectra was double-integrated (G) from 3405 to 3555 G to determine the total number of MTSL spins (○) and from 3443 to 3477 G to determine the spins derived from the strongly immobilized (a) and weakly immobilized (b) peaks (●).

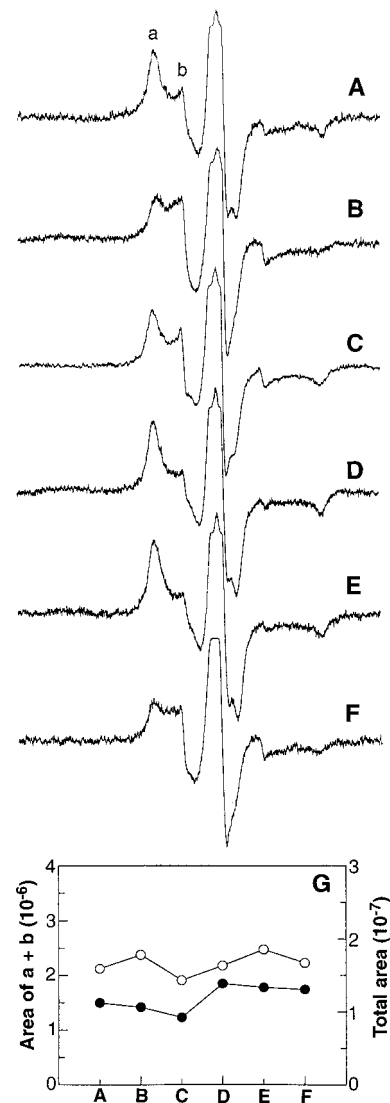


Fig. 3. Effect of colicin B on the mobility of MTSL attached to FepAE280C in live bacteria, observed in signal-averaged X-band spectra. KDF541/*pFepAE280C* was grown, spin-labeled, and prepared as described in Fig. 1. Signal-averaged X-band spectra (six field sweeps collected over an 8-min period) of spin-labeled bacteria were recorded in the presence of saturating levels of colicin B (10 μM) at 4°C (A). The sample was warmed to 37°C and analyzed after 5 (B), 15 (C), and 75 (D) min (midpoints of signal average measurement) before recooling to 4°C (E). The same strain was grown in minimal media and starved for glucose for 2 hours to deplete its energy stores, spin-labeled, and exposed to colicin B (10 μM) at 37°C for 30 min (F). The spectra were double-integrated (G) as described in Fig. 2.

sively eliminated weakly immobilized spins, enhanced the strongly immobilized peak, and increased the separation between low- and high-field extrema (Figs. 3 and 4). These changes did not result from colicin binding, because they did not take place during continual incubation of the cells at 4°C, even over the course of 1 hour. However, when cells with bound colicin were warmed to 37°C, MTSL at residue 280 showed a progressive immobilization that became more severe over 30 min, ultimately converting virtually all weakly immobilized spins to the strongly immobilized state (Figs. 3 and 4). As observed for the siderophore, these changes correlated with the transport phase of the colicin through the OM (24). Colicins presumably traverse the OM bilayer through porin channels (24), and the progressive immobilization of MTSL by colicin B may reflect steric restriction of spin-label motion as the colicin polypeptide passes through the FepA pore. The irreversibility of the immobilization during recooling supports the idea that the toxin polypeptide remains bound within FepA subsequent to OM transport. Both the kinetics and the nature of colicin-induced effects differed from the actions of ferric enterobactin, which probably reflects the different steric constraints placed on the

spin-labeled site during uptake of the two structurally diverse ligands.

Continuous monitoring of MTSL mobility (at 3472 G) over time showed that ferric enterobactin stimulated a burst of motion that peaked and receded over a 5-min period (Fig. 5). This surge in motion accrued from the sum of individual, weakly immobilized MTSL spins in the bacterial sample. The changing mobility of the spin label in PL5 very likely reflects the opening and closing of the gated FepA channel during siderophore uptake. Kinetic analysis of the spin-label motion tracing, which showed two peaks of mobility approximately 10 and 20 min after the temperature shift to 37°C, revealed in both cases a sigmoidal rise to a maximum followed by an approximately symmetrical decay to a minimum. The integrated areas beneath the two curves were the same (25), suggesting that the changes in motion arose from an initial, relatively synchronous transport cycle and a second, less synchronous cycle in the same number of cells. The first surge in motion peaked in 140 s and decayed in 140 s; because ferric enterobactin was supplied in excess (26), the decrease in motion did not result from its depletion. Thus, the time-resolved fluctuations in PL5 were consistent with an active opening and closing of the chan-

nel during the ferric enterobactin transport reaction (27).

The relocation of spin labels attached to E280C, by both ligands, showed the hallmarks of energy- and TonB-dependence: Glucose deprivation, low temperatures, and use of a *tonB* strain all prevented the effects (Fig. 5). During ligand internalization, FepA PL5 changed to either mobilize (by decreasing their contact with other components of FepA structure) or immobilize [by increasing their contact with the FepA polypeptide or the protein (colicin B) that interacted with it] attached spin labels. Hence, FepA fluctuates

Fig. 4. Statistical analysis of ferric enterobactin- and colicin B-induced variations in FepAE280C-MTSL motion. KDF541/pFepAE280C was grown, spin-labeled, and prepared as described in Fig. 1. Multiple signal-averaged spectra were collected as in Figs. 2 and 3 from several independent preparations of bacteria, and the mean and standard deviation of the ratio *b/a* was calculated at the indicated points after the addition of ferric enterobactin (A) (*n* = 4) and colicin B (C) (*n* = 6). The most sensitive measure of changes in spin-label mobility was the ratio of the amplitudes of *b/a*, shown and analyzed here. However, the effects of the siderophore and colicin were also analyzed by several other methods, including measurements of the absolute amplitudes, areas, separation of low- and high-field extrema ($2T_{1/2}$) and half-width at half-height (32) for peaks a and b. All these methods showed the same tendency of the siderophore to mobilize spin labels and the colicin to immobilize them. (A) Bars 1 through 6 correspond to samples A through F, respectively, of Fig. 2. (C) Bars 1, 2, 3, 5, and 6 correspond to panels A through E, respectively, of Fig. 3; bar 4 shows the effects of colicin after 30 min at 37°C. Superimposed spectra from single experiments are also shown in the region of the strongly and weakly immobilized peaks. (B) Spectra were collected in the absence of ferric enterobactin at 37°C (purple), in the presence of ferric enterobactin at 4°C (blue), after increasing the temperature to 37°C for 5 (green) and 15 (red) min, and after recooling the sample to 4°C (black). (D) Spectra were collected in the presence of colicin B at 4°C (purple), after increasing the temperature to 37°C for 5 (blue), 15 (green), and 75 (red) min, and after recooling the sample to 4°C (black).

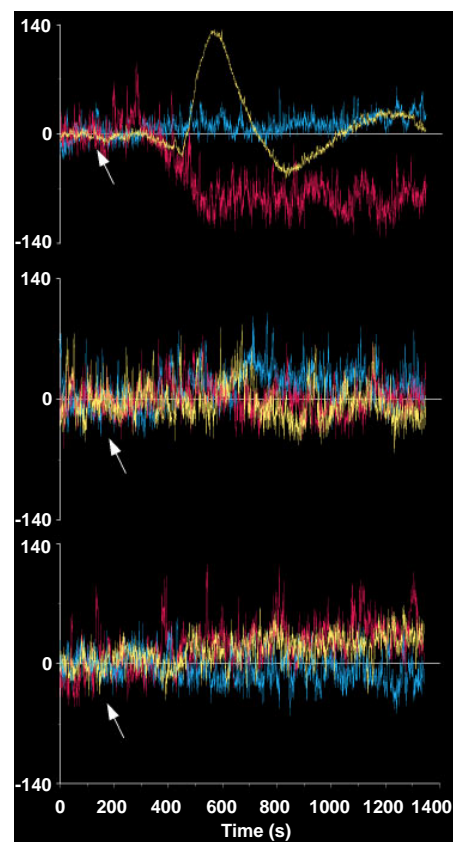
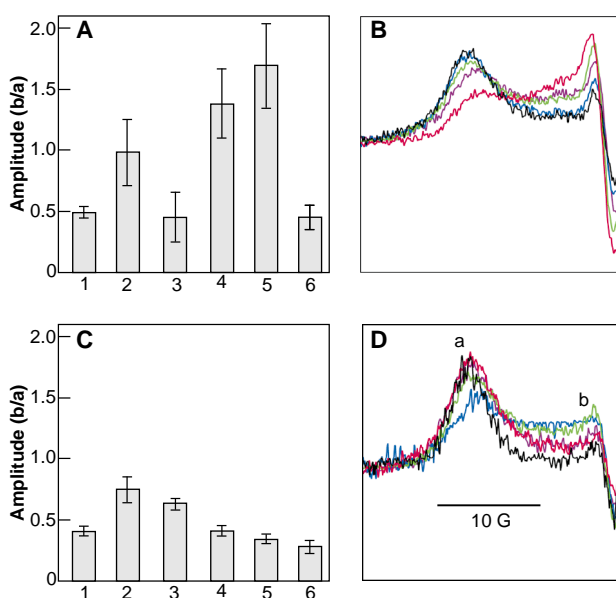


Fig. 5. TonB and energy dependence of conformational changes in FepAE280C. (Top) *fehA, tonB*⁺ bacteria (KDF541) expressing FepAE280C were spin-labeled with MTSL and analyzed in the presence of glucose. The intensity of the weakly immobilized peak (b in Fig. 1) was monitored versus time by setting the spectrometer center magnetic field at the peak maximum (3472.3 G) and the sweep width to zero. Bacteria were incubated at 4°C without added ligands (blue tracing) and warmed to 37°C after 5 min (marked by an arrow). The experiment was repeated in the presence of ferric enterobactin (300 μM; yellow) or colicin B (10 μM; red), as described in Figs. 2 and 3. (Center) *fehA, tonB*⁺ bacteria (KDF541) expressing FepAE280C were analyzed, but were energy-depleted by glucose starvation described in Fig. 3 and subjected to the conditions described above. (Bottom) Analysis of *fehA, tonB* bacteria [KDF570 (9)] expressing FepAE280C, subjected to the conditions described above.

between at least two conformations during ferric enterobactin transport, and colicin entry creates a different kind of conformational dynamic. The TonB- and energy-dependence of the observed structural changes confirms their physiological relevance (28), and the ligand-specific oscillation of probes attached to FepA PL5 suggests how siderophores enter the normally closed pores: TonB triggers energy-dependent structural changes in the ligand-binding site that displace PL5 and release the bound siderophore into the FepA channel. These results constitute physical evidence for the in vivo operation of a ligand-gated channel. They demonstrate what has been widely hypothesized, that gated porins open and close during solute transport. The perception of OM porins as rigid molecules containing passive channels, derived from crystallographic studies in vitro (4), contrasts with this characterization of FepA as a dynamic entity in vivo.

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- Microorganisms secrete low molecular weight organic molecules called siderophores (Greek for "iron carrier") in response to environmental iron deficiency. The native *E. coli* siderophore enterobactin forms a hexadentate complex with Fe³⁺ that is recognized and transported by most Gram-negative bacteria [J. B. Neilands, *Annu. Rev. Microbiol.* **36**, 285 (1982); *J. Biol. Chem.* **270**, 26723 (1995); J. M. Rutz, T. Abdullah, S. P. Singh, V. I. Kalve, P. E. Klebba, *J. Bacteriol.* **173**, 5694 (1991)].
- General porins, like *Rhodobacter capsulatus* porin [M. S. Weiss *et al.*, *FEBS Lett.* **267**, 268 (1990)]; M. S. Weiss *et al.*, *Science* **254**, 1627 (1991)] and *E. coli* OmpF [S. K. Cowan *et al.*, *Nature* **358**, 727 (1992)], form water-filled, transmembrane β barrels, with an approximate size-exclusion diameter of 10 Å [H. Nikaido and E. Rosenberg, *J. Gen. Physiol.* **77**, 121 (1981); *J. Bacteriol.* **153**, 241 (1981)].
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- In addition to an OM receptor protein, siderophore transport systems include proteins in the periplasm and inner membrane. TonB is the most notable of the accessory proteins; its NH₂-terminus resides in the cytoplasmic membrane, but downstream regions of its sequence may project across the periplasm and facilitate transport through OM receptors by direct, protein-protein interactions [for review, see R. J. Kadner, *Mol. Microbiol.* **4**, 2027 (1990); K. Postle, *ibid.*, p. 2019; V. Braun, K. Gunter, K. Hantke, *Biol. Met.* **4**, 14 (1991); P. E. Klebba, J. M. Rutz, J. Liu, C. K. Murphy, *J. Bioenerg. Biomembr.* **25**, 603 (1993)].
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- Examples include the recognition of vitamin B₁₂, bacteriophage BF23, and colicin E1 and E3 by BtuB [D. R. DiMasi *et al.*, *J. Bacteriol.* **115**, 56 (1973)]; the recognition of ferric enterobactin and colicins B and D by FepA [S. Guterman, *ibid.* **114**, 1217 (1973); A. P. Pugsley and P. Reeves, *ibid.* **126**, 1052 (1976)]; R. R. Wayne, K. Frick, J. B. Neilands, *ibid.*, p. 7]; the binding of ferriochrome; bacteriophages T1, T5, and ϕ 80; colicin M; and albomycin to TonA (FhuA) [R. Wayne and J. B. Neilands, *ibid.* **121**, 497 (1975)]; and the binding of colicins A and N to OmpF [H. Benedetti *et al.*, *J. Mol. Biol.* **217**, 429 (1991)].
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- FepAE280C contains the site-directed substitution of Cys for Glu at residue 280.
- At neutrality, MAL6 labels Cys residues about 1000-fold more efficiently than it does Lys; the specificity of MSL for Cys is essentially absolute [L. J. Berliner, *Ann. N.Y. Acad. Sci.* **414**, 153 (1983)].
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- The determination of labeling specificity presents an obstacle to the site-directed spin labeling of living cells. In the case of bacteria, however, the selectivity of chemical labeling can be evaluated by the use of null mutants [for example, FepA-deficient (*fepA*) bacteria; Fig. 1]. Furthermore, bacterial OM proteins contain few Cys residues, and available data suggest that those form disulfide bonds deep in tertiary structure [T. Schirmer, T. A. Keller, Y.-F. Wang, J. P. Rosenbusch, *Science* **267**, 512 (1995); (15)]. Therefore, in combination with site-directed mutagenesis to introduce unique Cys residues, spin labels can be attached and verified at sites of interest within membrane proteins of viable cells.
- Strongly immobilized spins probably result from steric hindrance of spin-label motion (rotation) by adjoining components of protein structure. Weakly immobilized spins, on the other hand, originate from the localization of the spin label in a less restricted environment, probably in better contact with the aqueous milieu. To determine the percentage of weakly immobilized spins, we double-integrated spectra in region of peaks a and b (3443 to 3477 G), using the Bruker WinEPR analysis software, and calculated the ratio of areas b/(a + b).
- The instantaneous fraction of weakly immobilized spins was not determined from X-band field sweeps (Fig. 2), because the ferric enterobactin transport reaction occurs much faster (8) than the time required (8 min) to collect the signal average of the six field sweeps shown in Fig. 2. Double integrations of the X-band spectra in Fig. 2 represent an average percentage of weakly or strongly immobilized spins, collected over an 8-min period.
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- MTSL-labeled FepAE280C bound and transported ferric enterobactin comparably to wild-type FepA [S. M. C. Newton and P. E. Klebba, unpublished data; (8, 17)].
- H. Benedetti, R. Llobes, C. Lazdunski, L. Letellier, *EMBO J.* **11**, 441 (1992); V. Braun, J. Frenz, K. Hantke, K. Schaller, *J. Bacteriol.* **142**, 162 (1980); D. Baty *et al.*, *Biochimie* **72**, 123 (1990); M. Wisner, D. Freymann, P. Ghosh, R. M. Stroud, *Nature* **385**, 481 (1997). Subsequent to OM penetration, pore-forming bacteriocins like colicin B remain associated with their OM receptors, but traverse the periplasm to form voltage-gated channels in the inner membrane that depolarize cellular energetics.
- Time-resolved changes in spin-label motion were analyzed and fitted with "Peak Fit" (Jandel Scientific, San Rafael, CA).
- The rate of ferric enterobactin uptake in these conditions is 1 nmol per minute per 10¹⁰ cells (8). In Figs. 2 and 4, 30 nmol of ferric enterobactin were added to the flat cell containing 10¹⁰ cells. Thus, even after 20 min in the presence of ferric enterobactin, the bacteria remained saturated with the siderophore.
- The properties of the fully induced ferric enterobactin transport system are: binding $K_d = 20$ nM, transport $K_m = 200$ nM, $V_{max} = 100$ pmol per minute per 10⁹ cells (8). In such iron-deficient conditions, the bacteria contained 80,000 to 100,000 FepA proteins per cell (8). The FepA turnover number for ferric enterobactin, calculated from these data as one molecule per minute per FepA monomer, compares well with the 5-min motion cycle seen in Fig. 5, especially because the transport measurements were made in exponentially growing bacteria. In the spectrometer cavity, the bacteria were rapidly shifted (in 90 s) from a state of dormancy at 4°C to active transport at 37°C.
- The requirements for energy and TonB excluded the possibility that the results derived from artifacts of the spin-labeling methodology or the application of this approach to living cells. Prior experiments on conformational changes in FepA associated with ferric enterobactin binding in vitro, using X-band spectra, were complicated by the fact that Fe³⁺ is itself paramagnetic, and may therefore undergo dipole-dipole interactions with the nitroxide spin labels. Dipolar effects from ferric iron, however, exert a broadening effect on nitroxide spectra, opposite to the results we observed in FepA in vivo. Such dipolar interactions, furthermore, are not expected to manifest either TonB- or energy dependence.
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- Bacteria in a 100- μ l quartz flat cell were cooled to 4°C or warmed to 37°C in the resonator cavity. The temperatures in the cavity were calibrated and monitored with a thermometer.
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- We thank Y. Kotake at the ESR Center of the Oklahoma Medical Research Foundation and E. E. Klebba for helpful discussions and J. Liu, C. Murphy, B. Kalyanaraman, and P. Cook for their comments on the manuscript. Supported by grants from NSF (MCB9212070 and CHE9512996 to P.E.K.) and NIH (GM53836 to P.E.K. and GM51339 to J.B.F.).

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