Biphase Binding Kinetics between FepA and Its Ligands*

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Like other TonB-dependent outer membrane proteins, FepA serves as a receptor for a metal chelate (ferric enterobactin (FeEnt)) and for noxious agents (colicin B (ColB) and colicin D (ColD)). Like other outer membrane (OM) porins (1–3), FepA contains a hydrophilic channel (4), but the FepA pore (5) is closed by cell surface loops that impart binding and translocation specificity. Thus FepA is a TonB-dependent, energy-dependent, ligand-gated porin: its surface loops open in response to ligand binding and TonB action, to internalize FeEnt (6).

The siderophore FeEnt and the cytotoxins ColB and ColD differ in size, structure, and uptake mechanism, yet all three siderophores bind to FepA and FepA mutants with colicin B-Sepharose. Analysis of a fluorescent FepA derivative showed that ferric enterobactin and colicin B adsorbed with biphase kinetics, suggesting that both ligands bind in at least two distinct steps, an initial rapid stage and a subsequent slower step, that presumably establishes a transport-competent complex.

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cyanoegen bromide (CNBr)-activated Sepharose 4B (21, 22). Crude or partially purified FepA was loaded on the column, which was washed with 10 volumes of TFE buffer (2% Triton X-100 in 50 mM Tris-Cl, 5 mM EDTA, pH 7.4), and FepA was eluted with a gradient of 0 to 2.5 mM NaCl in TFE. Fractions were analyzed by SDS-PAGE. We regeneranted the column with a reverse urea gradient (6.0 to 0.0 M in TFE).

α-FepA Sera—Rabbits were immunized weekly for 1 month with purified FepA, emulsified with complete (first immunization) or incomplete (subsequent immunizations) Freund’s adjuvant. Rabbits were bled in week 5 and thereafter. The IgG fraction of the resulting α-FepA serum was purified and conjugated to CNBr-activated Sepharose 4B (22).

Protein Determinations—Protein concentrations were determined by the method of Lowry et al. (23), modified for accuracy in the presence of Triton X-100 (24, 25). The concentration of ColB was determined by the absorbance at 280 nm using a molar extinction coefficient of 62,160 M⁻¹ cm⁻¹, calculated from its primary sequence (26).

**FeEnt Purification—**Fe-Enterobactin was prepared and purified as described previously (27).

ColB Killing Assays—Purified colicin was serially diluted in LB broth in microtiter plates, from 10⁻¹ to 3.2 · 10⁻⁹, and transferred with a sterile CloneMaster™ (Immuneus Corp., San Leandro, CA) to an LB plate containing ampicillin (10 μg/ml), seeded with the tester strain. Colicin titers were measured after overnight incubation, as the inverse of the highest dilution that cleared the bacterial lawn.

Preparation of 125I-ColB—ColB was iodinated with IODO-BEADS (Poly Scientific, Holmdel, NJ). Purified proteins were incubated with 500 μl of MOPS-buffered saline (MBS; 40 mM MOPS, 0.9% NaCl, pH 6.9), dried, and incubated, with 1 μCi of Na¹²⁵I in 300 μl of MBS for 5 min. 2.8 mg of ColB in 3.9 ml of MBS was added to 10 min at 25 °C. The reaction was stopped by removing the beads, and unrecovered 125I was eliminated by chromatography over Sephadex G-50 in MBS.

Fluorescence Measurements—The site-directed mutant FepA protein E280C was utilized for introduction of fluorescent probes onto the receptor. This mutant protein has been extensively studied and manifests a wild-type phenotype, even when covalently modified at the E280C site (6, 10, 12, 31). Its structural integrity in vivo was assessed with a battery of tests including accessibility to monoclonal antibodies, expression levels, binding and transport of ferric enterobactin, and binding and killing by colicins B and D. Purified FepAE280C-fluorescein bound ferric enterobactin with a Kd almost identical to the wild type FepA, providing good evidence that the overall structure of E280C was intact. We cannot completely rule out that fluorescent labeling may have some impact on the binding of ligands, but based on the above studies, we believe it is minimal. FepA-E280C-Fl fluorescence expression levels, binding and transport of ferric enterobactin, and accessibility to monoclonal antibodies, expression levels, binding and transport of ferric enterobactin, and binding and killing by colicins B and D. Purified FepA-E280C-fluorescein was intact. We cannot completely rule out that fluorescent labeling may have some impact on the binding of ligands, but based on the above studies, we believe it is minimal.

FeEnt and ColB Binding Equilibria in Vivo—FepA in RWB1860/pITS449, cultured under iron stress in MOPS medium, bound FeEnt with a Kd of 24 ± 8 nM to a capacity 103,000 molecules/cell (Fig. 1). The siderophore reached equilibrium with FepA within 1 min. The affinity of the receptor for colicins was somewhat lower; it bound ColB with a Kd of 185 ± 45 nM, to a capacity of 96,000 molecules/cell (Fig. 1). Measurements of

**FepA expression in 125I-protein A Western blots (75,000 monomers/cell; data not shown) were consistent with the observed siderophore and ColB binding capacities, indicating approximately 1:1 stoichiometry for the binding of either ligand to the FepA monomer. FepA bound ColD, under the same experimental conditions, with a Kd of 560 ± 130 nM, to a capacity of 33,000 molecules/cell, 3-fold lower than ColB (Fig. 1). Colicin binding experiments were incubated from 10 min to 3 h with no significant change in the parameters (data not shown), indicating that the reactions were at or near equilibrium.

In Vivo Competition between FeEnt and ColB for Binding to FepA—A 10-min preincubation of bacteria with saturating FeEnt (8.2 μM), or inclusion of saturating siderophore in the 125I-ColB binding assay, prevented ColB binding (Fig. 2A). These data reiterate that FeEnt protects E. coli from ColB killing by occupancy of a common binding site on FepA (29, 30).

**RESULTS**

**FeEnt and ColB Binding Equilibria in Vivo—**FepA in R WB1860/pITS449, cultured under iron stress in MOPS medium, bound FeEnt with a Kd of 24 ± 8 nM to a capacity 103,000 molecules/cell (Fig. 1). The siderophore reached equilibrium with FepA within 1 min. The affinity of the receptor for colicins was somewhat lower; it bound ColB with a Kd of 185 ± 45 nM, to a capacity of 96,000 molecules/cell (Fig. 1). Measurements of

**Fig. 1 Ligand binding curves.** Data were obtained with RWB18–60 (pfaA) (11) and RWB18–60/pITS449 (pfaA+) (11) (FeEnt) (12), binding to RWB18–60 was subtracted from each point. Theoretical binding curves are superimposed on the data. [FeEnt] (τ), average of three experiments), the Kd values of 45 ± 8 nM to a capacity of 7 pmol/10⁶ cells. [ColB] (τ, average of three experiments), the Kd values of 185 ± 45 nM and the capacity 96 ± 3 pmol/10⁶ cells. [ColD] (τ, average of two experiments), the Kd values of 560 ± 130 nM and capacity 33 ± 3 pmol/10⁶ cells.

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To further study the slow release of ColB from FepA, we used conditions that prevented its reabsorption, saturating FeEnt. We equilibrated bacteria with 3.5 μM 125I-ColB, then diluted, pelleted, and resuspended the cells in buffer with 10 μM FeEnt. From the radioactivity of the pellet, measured at intervals, we calculated koff for ColB, 4.7 × 10⁻⁵ s⁻¹, a half-life (45) of 4 h. The rate of 125I-FeEnt release under the same conditions was 6.9 × 10⁻⁴ s⁻¹, a half-life of about 20 min. While the slow off-rates explained the ligand competition results, they were inconsistent with a one-step binding mechanism; assuming single-step ColB binding and a kobs of 10⁸ M⁻¹ s⁻¹ (an order of magnitude slower than the diffusion limit for small molecules), the measured Kd of 185 nM predicted a dissociation time of less than 1 s. The slower observed ligand dissociation rates suggested that the measured Kd values contained rate constants for more than one binding step. The simplest explanation was that ColB bound in two steps, a rapid initial phase and a second phase that locked the receptor and ligand into a slow dissociating complex.
Ligand Binding to a Gated Porin

In vitro competition between $^{125}$I-ColB and FeEnt. Binding was measured as in Fig. 1 except that $8.2 \mu M$ FeEnt was included in the assays, creating a final volume of 70 $\mu l$. Binding to RWB18–60 was subtracted from each point. A. FeEnt was added and incubated for 10 min, followed by $^{125}$I-ColB and further incubation for 1.0 h (●); the lines are a point by point connection of the data. Control binding curves were obtained by adding phosphate buffer in place of FeEnt (□): the solid line is the theoretical binding curve. The $K_d$ was $163 \pm 3$ nM, and the binding capacity was $91 \pm 3$ pmol/10$^9$ cells. B, $^{125}$I-ColB was added and incubated for 1.0 h, followed by FeEnt and further incubation for 10 min (●); the dashed line is the theoretical binding curve. The $K_d$ was $118 \pm 37$ nM, and the capacity was $91 \pm 3$ pmol/10$^9$ cells. A control binding curve was obtained by adding phosphate buffer in place of FeEnt (□): the solid line shows the theoretical binding curve. The $K_d$ was $212 \pm 38$ nM, and the capacity was $98 \pm 3$ pmol/10$^9$ cells.

In Vitro Ligand Binding in DM—The mutant protein FepAE280C (6, 10, 12, 31) binds and transports FeEnt, ColB, and ColD at wild type levels. Residue 280 lies in a proposed surface loop that participates in ligand binding (9, 10). We derivatized FepAE280C with the sulfhydryl-specific reagent 5-iodoacetamidofluorescein. The fluorescence intensity of the derivatized FepAE280C with the sulfhydryl-specific reagent 5-iodoacetamidofluorescein was $37 $nM, and the capacity was 98 $pmol/10^9$ cells.

In vivo competition between $^{125}$I-ColB and FeEnt. The decrease in fluorescence intensity after addition of FeEnt is plotted versus total input FeEnt. In the absence of ColB, the $K_d$ was $15 \pm 3$ nM and the maximum fluorescence change (△$F$) was $0.41 \pm 0.01$. The line shows the theoretical binding curve. A 15-min preincubation with 2.0 $\mu M$ ColB (●) prevented FeEnt adsorption. The line is a point to point connection. Data points were corrected for dilution of the fluorophore by addition of titrant.

The reduction in FepAE280C-Fl fluorescence that occurred upon binding of ColB from purified FepA, demonstrating that the long lived

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FIG. 2. In vivo competition between $^{125}$I-ColB and FeEnt. Binding was measured as in Fig. 1 except that $8.2 \mu M$ FeEnt was included in the assays, creating a final volume of 70 $\mu l$. Binding to RWB18–60 was subtracted from each point. A. FeEnt was added and incubated for 10 min, followed by $^{125}$I-ColB and further incubation for 1.0 h (●); the lines are a point by point connection of the data. Control binding curves were obtained by adding phosphate buffer in place of FeEnt (□): the solid line is the theoretical binding curve. The $K_d$ was $163 \pm 3$ nM, and the binding capacity was $91 \pm 3$ pmol/10$^9$ cells. B, $^{125}$I-ColB was added and incubated for 1.0 h, followed by FeEnt and further incubation for 10 min (●); the dashed line is the theoretical binding curve. The $K_d$ was $118 \pm 37$ nM, and the capacity was $91 \pm 3$ pmol/10$^9$ cells. A control binding curve was obtained by adding phosphate buffer in place of FeEnt (□): the solid line shows the theoretical binding curve. The $K_d$ was $212 \pm 38$ nM, and the capacity was $98 \pm 3$ pmol/10$^9$ cells.

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The reduction in FepAE280C-Fl fluorescence that occurred upon binding of FeEnt or ColB followed a double exponential decay (Fig. 4). Convergence did not occur with a single exponential decay model. Fluorescence decreased during FeEnt binding (Fig. 4B) with an initial rapid phase ($k_1$: $1.8 \times 10^{-2} \pm 8 \times 10^{-4} s^{-1}$; see “Discussion” for rate constant nomenclature) and a second slower phase ($k_2$: $2.1 \times 10^{-3} \pm 2 \times 10^{-3} s^{-1}$). ColB binding also followed biphasic kinetics (Fig. 4A) with faster initial ($k_3$: $2.1 \times 10^{-2} \pm 1 \times 10^{-3} s^{-1}$) and a slower secondary ($k_4$: $3 \times 10^{-4} \pm 1 \times 10^{-4} s^{-1}$) components. Thus FeEnt and ColB both exhibited complex binding and a similar rate of initial adsorption to purified FepA in DM; their secondary, slower binding components differed by about 10-fold.

Ligand Dissociation in DM—We also saw slow dissociation of ColB from purified FepA, demonstrating that the long lived

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complex depends solely on the receptor and ligand; other OM components or FepA localization in the OM were not required. When FepAE280C-Fl-ColB was incubated with excess FeEnt over an extended period, a further decrease in fluorescence gradually occurred (Fig. 5) that followed a simple exponential decay. It was slower \( k_{\text{off}} = 6.7 \times 10^{-4} \pm 4 \times 10^{-6} \text{ s}^{-1} \), than the predicted off-rate, but 10-fold faster than ColB release in the absence of FeEnt in vitro. This decrease in fluorescence reflected the conversion of the FepA-ColB complex to the FepA-FeEnt complex. It occurred slower than the rate of FeEnt binding and probably approximates \( k_{\text{off}} \) for ColB in vitro. Likewise, addition of excess ColB to FepAE280C-Fl-FeEnt slowly increased fluorescence intensity (Fig. 6), again by a first order exponential process \( k_{\text{on}} = 3.9 \times 10^{-3} \pm 6 \times 10^{-5} \text{ s}^{-1} \), suggesting that its significant overall charge \((-3)\) and aromaticity outweigh the potential for multivalent charge interactions between the colicins and the receptor. FeEnt may bind in a pocket of the receptor surface that is optimized to accept it in a complementary configuration. The colicins also use this site, but apparently as part of a broader surface area that creates weaker overall interactions. The lower capacity of FepA for ColD, relative to that observed for ColB and FeEnt, suggests that the larger colicin may bind as 1 ColD molecule per FepA trimer, as opposed to 1 molecule per FepA monomer, seen for ColB and FeEnt.

**Ligand Binding in Triton X-100**—Ligand binding kinetics in 2% Triton X-100 were similar to those observed in DM (Table I), except that in Triton X-100 the fluorescence of FepAE280C-Fl increased approximately 15% upon ColB addition. Nevertheless, the fluorescence of FepAE280C-Fl in Triton X-100 decreased approximately 30% upon the addition of FeEnt, consistent with the results in DM, and the antagonistic behavior of the two ligands in Triton X-100 was the same as was seen in DM. The rates of fluorescence changes in Triton X-100 were very similar to those measured in DM (Table I), and in both detergents ligand binding was clearly a two-component process (see Scheme 1).

**Affinity Purification of FepA**—The avidity of FepA for its ligands suggested affinity chromatography as a method for its purification. ColB-Sepharose purified FepA from Triton X-100-solubilized OM fractions of RWB18–60/pFepAE280C (Fig. 7A). It was more effective than immunoaffinity chromatography (Fig. 7B), because 81Kx (32), the OmpT-generated degradation product of FepA, stuck tightly to anti-FepA-Sepharose, but not to ColB-Sepharose. Second, a mild salt gradient released FepA from ColB-Sepharose, while only high concentrations of the chaotropic agent trichloroacetate eluted the receptor from the immunoadsorbent, and these harsh conditions also released antibodies from the resin that contaminated the FepA product.

**DISCUSSION**

Affinity purification of siderophore receptors by binding to their antagonistic ligands, bacteriocins, was reported by Oudega et al. (21), who used cloacin DF13-Sepharose to purify an OM protein later recognized as the ferric aerobactin receptor, IutA (33). We reproduced this technique with ColB-Sepharose, permitting large scale, rapid purification of FepA or mutant FepA proteins; 20 ml of the affinity adsorbent isolated 5–10 mg of the receptor in 1–2 days. Guterman (29, 34) discovered two mutations, exbA and exbB, that resulted in ColB resistance and hyperexcretion of a diffusible inhibitor of ColB killing. It was later recognized as tonB (35), which creates OM transport deficiencies resulting in ColB resistance. exbB also produces colicin uptake defects, and the soluble factor was identified as FeEnt. tonB and exbB mutants secrete enterobactin because they are unable to transport iron, making them chronically iron-deficient (36). Later experiments showed that metal chelates (30, 37, 38) block colicin (and bacteriophage) killing by competitive binding to common surface receptors. FeEnt, ColB, and ColD, for example, adsorb to a common site in FepA PL5 (10). We measured the affinities and kinetics of these binding reactions in vivo and in vitro. The siderophore bound more tightly to FepA than ColB or ColD, suggesting that its significant overall charge \((-3)\) and aromaticity outweigh the potential for multivalent charge interactions between the colicins and the receptor. FeEnt may bind in a pocket of the receptor surface that is optimized to accept it in a complementary configuration. The colicins also use this site, but apparently as part of a broader surface area that creates weaker overall interactions. The lower capacity of FepA for ColD, relative to that observed for ColB and FeEnt, suggests that the larger colicin may bind as 1 ColD molecule per FepA trimer, as opposed to 1 molecule per FepA monomer, seen for ColB and FeEnt.

In the two nonionic detergents we employed FeEnt and ColB engendered spectroscopically distinct states in FepA upon binding, suggesting that the siderophore and colicins induce different effects when they complex FepA. Conformational changes in FepA were previously observed in the presence of FeEnt, by analysis of nitroxide probes attached at E280C (12). The close proximity of E280C to the FepA ligand binding domain (12) raises the possibility that the large reduction in intensity caused by the ferric siderophore results from chemical quenching instead of conformational changes in the receptor. However, both FeEnt and ColB exhibited biphasic binding kinetics to FepA, which strongly suggests a conformational change in the receptor during their adsorption. Analyses of the two-component pseudo-first order decay of fluorescent probes attached in PL5 (Table I) revealed several possible equilibria.
Ligand Binding to a Gated Porin

Table I
Summary of rate constants

| Rate constant | Value | S.E. | $t_{1/2}$ | Value | S.E. | $t_{1/2}$ |
|---------------|-------|------|----------|-------|------|----------|
| $k_1$         | 1.8 x 10^{-2} | 8 x 10^{-4} | 0.65 min | 6.4 x 10^{-3} | 3 x 10^{-4} | 1.8 |
| $k_2$         | 2.1 x 10^{-3} | 2 x 10^{-4} | 5.5 min | 1.15 x 10^{-3} | 7 x 10^{-4} | 10.0 |
| $k_3$         | 2.1 x 10^{-2} | 1 x 10^{-3} | 0.55 min | 8.9 x 10^{-3} | 5 x 10^{-4} | 1.3 |
| $k_4$         | 3 x 10^{-2} | 1 x 10^{-3} | 39 min | 1.09 x 10^{-3} | 3 x 10^{-5} | 10.6 |
| $k_5$         | 3.9 x 10^{-3} | 6 x 10^{-5} | 3 min | 2.1 x 10^{-3} | 6 x 10^{-6} | 5.5 |
| $k_6$         | 6.7 x 10^{-3} | 4 x 10^{-6} | 17 min | 8.6 x 10^{-4} | 2 x 10^{-6} | 13.4 |
| $k_{ad}$ FeEnt<sup>a</sup> | 6.9 x 10^{-4} | ND<sup>b</sup> | 17 min |       |       |         |
| $k_{ad}$ ColB<sup>d</sup> | 4.7 x 10^{-5} | ND<sup>b</sup> |       |       |       | (3 min<sup>c</sup>) |

<sup>a</sup> Determined in 40 mM MOPS, pH 6.9, containing 0.9% NaCl, 2 μM nonradioactive FeEnt, and no detergent, 0 °C.
<sup>b</sup> ND, not determined.
<sup>c</sup> Extrapolated to 25 °C.
<sup>d</sup> Determined in 40 mM MOPS, pH 6.9, containing 0.9% NaCl, 10 μM FeEnt, and no detergent, 0 °C.

(Scheme 1). In DM the initial, fast binding component was similar for both ligands (approximately 0.02 s<sup>-1</sup>), and the secondary, slow component was about 10-fold faster for FeEnt (2.1 x 10<sup>-3</sup> s<sup>-1</sup>) than ColB (3 x 10<sup>-4</sup> s<sup>-1</sup>). In Triton X-100 the binding of both ligands was also biphasic, but the initial stages were slower (approximately 0.006 s<sup>-1</sup>), and we did not observe rate differences in the second stages of FeEnt and ColB binding (Table I). These data suggest a detergent effect on the binding reaction (see also below).

The mutual exclusion of one ligand by the binding of the other allowed us to measure their dissociation rates from FepA. In contrast to the bifasic association kinetics observed for FeEnt and ColB, their dissociation rates in the presence of the other followed single-component first order decays. The release of the siderophore and the binding of the colicin, or vice versa, may follow one of two pathways. In a microscopically reversible model, the two-stage FeEnt binding pathway may simply revert to release the siderophore and free FepA for ColB binding. In contrast to the rate constant for release of FeEnt in the presence of ColB ($k_6$), this reflects the rate-limiting steps leading to FeEnt binding ($k_4$ and $k_5$) followed by the forward progress of ColB binding ($k_9$ and $k_7$). Then, $k_9$ reflects the rate-limiting step among $k_4$, $k_5$, $k_1$, and $k_2$. If the ligand exchange reaction proceeds along this sequential pathway, then the individual steps must occur as rapidly as the overall dissociation rate (i.e. $k_4$, $k_5$, $k_1$, and $k_2$ must be >> $k_9$). This condition was met in Triton X-100, but not in DM ($k_7$ was << $k_9$). A possible explanation for this difference is that in DM the rapid release of FeEnt from FepA ($k_9$) results from an active solubilization of the siderophore off the surface of the receptor by the detergent. For release of ColB in the presence of FeEnt, $k_{10}$ reflects the rate-limiting step among $k_6$, $k_9$, $k_1$, and $k_2$. In this case the individual steps occurred as rapidly as the observed dissociation rate ($k_6$, $k_9$, $k_1$, and $k_2$ were >> $k_{10}$), in both DM and Triton X-100. Thus, the microscopically reversible pathway accounts for the dissociation kinetics of both ligands in Triton X-100, and the release of ColB in DM is, and is the most likely explanation of the observed dissociation kinetics in both detergents. An alternative mechanism for the rapid release of FeEnt in the presence of ColB involves the binding of the toxin to FepA<sup>FeEnt</sup>, creating a ternary complex (FepA<sup>FeEnt-ColB</sup>) that dislodges the siderophore. Our data tends to exclude this mechanism, because no ColB binding was seen in the presence of FeEnt. Neither did we obtain evidence of a ternary complex in vitro, in <sup>59</sup>FeEnt binding experiments in the presence of saturating ColB (data not shown). Thus, our results are most consistent with full release of one ligand before binding of another.

One or more intermediate states must exist in the transport mechanism of a ligand-gated channel like FepA, in which the solute initially binds to an external region of the receptor. Our results show the existence of a second phase in the initial adsorption stage, that is energy- and TonB-independent, and precedes the TonB-dependent internalization of ligands. The likely explanation is that a conformational change occurs in FepA as a second step of binding, as previously suggested by site-directed spin labeling of FepA in vitro (12). Such an intermediate complex also explains the discrepancy between the $K_d$ of FeEnt binding (200 nM) and the $K_m$ of FeEnt transport (200 nM) (10); when an initial rapid binding occurs, followed by other intermediate steps, the $K_m$ deviates from the $K_d$ according to the rate constants for formation and collapse of the intermediates. In subsequent stages the conformation of FepA surface loops change to "open" the receptor, and the ligand enters the underlying channel (6). This internalization reaction, which may itself contain numerous distinct steps, ultimately requires energy expenditure and the participation of TonB to reach completion.

Like the siderophore, transport of the colicin occurs in two stages: binding to the cell surface, and passage through the OM bilayer. Again, our data address the energy-independent, biphasic first stage of adsorption to FepA. The steps after binding remain ill defined. FepA-ColB dissociates more slowly than FepA<sup>FeEnt</sup>; this slow dissociating complex likely results from conformational changes in the receptor and/or the ligand that hold the proteins together. The progression of ligand binding to
this tight complex did not require TonB or other OM components. Subsequent, TonB-dependent conformational changes in FepA likely trigger uptake after binding, by initiating colicin passage through the FepA channel (6).

**REFERENCES**

1. Weiss, M. S., Abele, U., Weckesser, J., Welte, W., Schiltz, E., and Schulz, G. E. (1991) Science 254, 1627–1630
2. Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Feix, J. B., and Klebba, P. E. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10653–10657
3. Davies, J. K., and Reeves, P. (1975) J. Bacteriol. 122, 1922–1928
4. Newton, A., and Neilands, J. B. (1975) J. Bacteriol. 126, 1149–1155
5. Timmis, K. (1972) J. Bacteriol. 111, 138, 14236–14239
6. Benedetti, H., Fre Nette, M., Baty, D., Loubilas, K., Knibiehler, M., Pattus, F., and Lazdunski, C. (1991) J. Mol. Biol. 217, 429–439
7. Timmis, K. (1972) J. Bacteriol. 109, 12–20
8. Pugsley, A. P., and Reeves, P. (1977) Antimicrob. Agents Chemother. 11, 345–353
9. Schnaitman, C. A. (1971) J. Bacteriol. 108, 553–563
10. Lluelles, R., Knibiehler, M., Abdulla, T., Singh, S. P., and Pattus, F. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10675–10679
11. Armstrong, S. K., Francis, C. L., and McIntosh, M. A. (1990) J. Mol. Biol. 217, 429–439
12. Liu, J., Rutz, J. M., Feix, J. B., and Klebba, P. E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10653–10657
13. Wiener, M., Freymann, D., Ghosh, P., and Stroud, R. M. (1997) Nature 385, 461–464 (see comment in Nature (1997) 385, 390–391)
14. Lazdunski, C. J. (1995) Mol. Microbiol. 16, 1059–1066
15. Jeanteur, D., Schirmer, T., Foureil, D., Simonet, V., Rummel, G., Widmer, C., Rosenbusch, J. P., Pattus, F., and Pages, J. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10675–10679
16. Benedetti, H., Fre Nette, M., Baty, D., Loubilas, K., Knibiehler, M., Pattus, F., and Lazdunski, C. (1991) J. Mol. Biol. 217, 429–439
17. Timmis, K. (1972) J. Bacteriol. 109, 12–20
18. Pugsley, A. P., and Reeves, P. (1977) Antimicrob. Agents Chemother. 11, 345–353
19. Schnaitman, C. A. (1971) J. Bacteriol. 108, 553–563
20. Fiss, E. H., Stanley-Samuelson, P., and Neillands, J. B. (1982) Biochemistry 21, 4517–4522
21. Oudega, B., Oldenziel-Werner, W. J., Klaassen-Boor, P., Reze, A., Glas, J., and de, G. P. K. (1979) J. Bacteriol. 138, 7–16
22. Mishell, B. B., and Shig, S. M., eds. (1980 Selected Methods in Cellular Immunology, W. H. Freeman, San Francisco
23. Lowry, O. H., Rosebough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 285–295
24. Wang, C., and Smith R. L. (1975) Anal. Biochem. 63, 414
25. Dulley, J. R., and Grieve, P. A. (1975) Anal. Biochem. 64, 136–141
26. Mende, J., and Braun, V. (1990) Mol. Microbiol. 4, 1523–1533
27. Rutz, J. M., Abdulla, T., Singh, S. P., Kalve, V. I., and Klebba, P. E. (1991) J. Bacteriol. 173, 5964–5974
28. Leatherbarrow, R. J. (1992) Grafit Version 3.0, Erathicus Software Ltd., Staines, UK
29. Guterman, S. K. (1971) Biochem. Biophys. Res. Commun. 44, 1149–1155
30. Wayne, R., Frick, K., and Neillands, J. B. (1976) J. Bacteriol. 126, 7–12
31. Klug, C. S., Su, W., Liu, J., Klebba, P. E., and Feix, J. B. (1995) Biochemistry 34, 14230–14236
32. Hollifield, W. C., Jr., and Neillands, J. B. (1978) Biochemistry 17, 1922–1928
33. Carbonetti, N. H., and Williams, P. H. (1984) Infect. Immun. 46, 7–12
34. Guterman, S. K. (1973) J. Bacteriol. 114, 1217–1224
35. Rutten, S. K., and Danz, L. (1973) J. Bacteriol. 114, 1225–1230
36. Wang, C. C., and Smith R. L. (1975) J. Bacteriol. 126, 285–295
37. Dhaas, D. R., White, J. C., Roberts, C. A., and Bradbeer, C. (1973) J. Bacteriol. 115, 514–521
38. Wayne, R., and Neillands, J. B. (1975) J. Bacteriol. 121, 497–503

**FIG. 7. SDS-PAGE of affinity purification of FepA.** A, affinity chromatography of crude FepA on ColB-Sepharose. OM fractions from KDF541 (4)pFepA280C were solubilized with TTE buffer (lane 1) and adsorbed to ColB-Sepharose in TTE. The column was washed with 10 volumes of buffer (lanes 2 and 3) and eluted with a gradient of 0.0 to 2.0 M NaCl in TTE (lanes 4–13). Purified ColB (lane 14) was utilized for preparation of ColB-Sepharose, and 125I-ColB. B, affinity chromatography of crude FepA on rabbit α-FepA-Sepharose. OM fractions from BN1071 (fepA+9) were solubilized with TTE (lane 1) and adsorbed to the resin. The column was washed (lanes 2–4) and eluted with a gradient of 0.0 to 2.0 M neutralized trichloroacetic acid in TTE (lanes 5–14). Arrows mark the positions, in descending order, of FepA, 81K*, Cir, OmpC, and OmpA.