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Transport of iron across the outer membrane

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Summary. The TonB protein is involved in energy-coupled receptor-dependent transport processes across the outer membrane. The TonB protein is anchored in the cytoplasmic membrane but exposed to the periplasmic space. To fulfill its function, it has to couple the energy-providing metabolism in the cytoplasmic membrane with regulation of outer membrane receptor activity. Ferrichrome and albomycin transport, uptake of colicin M, and infection by the phages T1 and $\Phi 80$ occur via the same receptor, the FhuA protein in the outer membrane. Therefore, this receptor is particularly suitable for the study of energy-coupled TonB-dependent transport across the outer membrane. Ferrichrome, albomycin and colicin M bind to the FhuA receptor but are not released into the periplasmic space of unenergized cells, or *tonB* mutants. In vivo interaction between FhuA and TonB is suggested by the restoration of activity of inactive FhuA proteins, bearing amino acid replacements in the TonB box, by TonB derivatives with single amino acid substitutions. Point mutations in the *fhuA* gene are suppressed by point mutations in the *tonB* gene. In addition, naturally occurring degradation of the TonB protein and its derivatives is preferentially prevented in vivo by FhuA and FhuA derivatives where functional interaction takes place. It is proposed that in the energized state, TonB induces a conformation in FhuA which leads to the release of the FhuA-bound compounds into the periplasmic space. Activation of FhuA by TonB depends on the ExbBD proteins in the cytoplasmic membrane. They can be partially replaced by the TolQR proteins which show strong sequence similarity to the ExbBD proteins. A physical interaction of these proteins with the TonB protein is suggested by TonB stabilization through ExbB and TolQR. We propose a permanent or reversible complex in the cytoplasmic membrane composed of the TonB protein and the ExbBD/TolQR proteins through which TonB is energized.

Key words: Iron – Outer membrane – *Escherichia coli* – TonB – FhuA – Colicin

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Translocation of compounds across the outer membrane

Escherichia coli, as a Gram-negative bacterial cell, is surrounded by two membranes, an outer membrane and an inner (cytoplasmic) membrane. The outer membrane is composed of a lipid bilayer with a high content of proteins. The amounts of individual proteins is very different ranging over 1000–100000 copies/cell (Lugtenberg and van Alphen 1983). The rate of protein synthesis varies strongly depending on the growth conditions. Regulation takes place mainly at the level of transcription initiation frequency. Substrates to be taken up into the cells have to be translocated across the outer membrane and the cytoplasmic membrane. Small hydrophilic substrates, up to a molecular mass of 600 Da, diffuse through water-filled channels formed by the porins. Facilitated diffusion with a certain degree of transport specificity is observed in the uptake of maltodextrins, phosphates and nucleosides. Selective uptake comes from the stereospecific recognition of the substrates by the transport proteins. Interestingly, these proteins are subject to regulation by the substrates delivered in the growth medium. Maltodextrins induce transcription of the *lamB* gene, and nucleosides of the *tsx* gene, while phosphate starvation enhances transcription of the *phoE* gene. However, these proteins are dispensable for growth on these substrates, provided the concentration of the substrates is sufficiently high and their molecular mass does not exceed 600–700 Da. Under these conditions, these substrates diffuse through the porin channels with sufficiently high rates to support growth.

In contrast, no uptake of iron(III) siderophores (and impaired uptake of vitamin B_{12}) is observed into cells devoid of outer membrane receptor proteins (Table 1). Their transport across the outer membrane is energy-coupled and requires recognition of the iron(III)

Table 1. TonB-dependent transport across the outer membrane

Compound	Outer membrane receptor
Fe^{3+}-aerobactin	IutA
Fe^{3+}-ferrichrome	FhuA
Albomycin	FhuA
Fe^{3+}-coprogen	FhuE
Fe^{3+}-rhodotorulic acid	FhuE
Fe^{3+}-dicitrate	FecA
Fe^{3+}-enterochelin (enterobactin)/DBS[b]	FepA
Fe^{3+}-cephalosporin catecholates/DBS	Fiu
Fe^{3+}-cephalosporin catecholates/DBS	Cir
Rifamycin (CEP4832)	FhuA
Vitamin B_{12}	BtuB
Colicin B	FepA
Colicin D	FepA
Colicin M	FhuA
Colcins Ia, Ib	Cir
Phage T1[a]	FhuA
Phage $\Phi 80$[a]	FhuA

[a] The phages adsorb irreversibly accompanied by phage DNA uptake
[b] DBS, dihydroxybenzoyl-serine

siderophores by the related receptor proteins. Binding of the substrates to the receptors occurs also in energy-deprived cells, so that the vectorial release from the receptors into the periplasmic space (located between the outer membrane and the cytoplasmic membrane) seems to be the energy-consuming step. However, no energy-providing system nor energy-rich metabolites are known to exist in the outer membrane or in the periplasmic space. Energy is generated in the cytoplasm and the cytoplasmic membrane, and charged molecules such as ATP and phosphoenol pyruvate are not exported. A way out of this dilemma was the observation that import processes, requiring cell-surface receptors and energy, have a common need for the *tonB* gene product. Mutants in the *tonB* gene are devoid of iron(III) siderophore uptake and are resistant to certain bacteriophages and colicins which use the same receptors as the iron(III) siderophores (Table 1). In the following discussion, data will be provided which point to a complex uptake mechanism across the outer membrane. This has lead to a completely revised view of the outer membrane, which formerly was considered to be nothing more than a protective skin. The term cell wall, still in use, reflects the old concept.

TonB protein for outer-membrane transport

The *tonB* gene locus was originally related to phage T1 (ton = T-one) infection in that *tonB* mutants were resistant to the phage. T1 reversibly adsorbed to *tonB* mutants without release of DNA from the phage head and uptake into the cytoplasm. Irreversible adsorption accompanied by infection required in addition cellular energy, which could be generated by the membrane-bound electron transport chain or by ATP hydrolysis (Hancock and Braun 1976). DNA uptake was independent of the TonB function since spontaneous phage mutants with an extended host range infected *tonB* mutants. These phage variants irreversibly bound to the isolated receptor protein designated FhuA (former designation TonA defining the second known gene for T1 infection), in contrast to wild-type phage which adsorbed only reversibly (Hantke and Braun 1978). It was concluded that the phage host-range mutants recognized a conformation of the FhuA receptor, which for the wild-type phage only serves for reversible adsorption but does not trigger a conformational change in the phage required for DNA release from the phage head. Apparently, the adsorption rather than the uptake of phage DNA required the TonB protein (and cellular energy).

The second line of evidence in this system came from studies concerned with the uptake of colicin M. This colicin binds to the same FhuA receptor as phage T1. It binds and stays bound to the receptor in *tonB* mutant cells or in unenergized cells (Braun et al. 1980). Mutants in *tonB* became colicin-M-sensitive only upon osmotic shock treatment which renders the outer membrane temporarily permeable. This result suggested that colicin M transport across the outer membrane depends on the TonB protein.

In addition to the polymeric phages and colicins, substances of low molecular mass are transported across the outer membrane by an energy- and TonB-dependent mechanism. It has recently been shown that ferrichrome, aerobactin and coprogen bind to the periplasmic FhuD transport protein (Köster and Braun 1990; see also Köster, this issue). In order to gain access to the FhuD protein, ferrichrome has to cross the outer membrane. No binding to FhuD was demonstrated in *tonB* (and *fhuA*) mutants, showing that the FhuA and TonB proteins are required for ferrichrome uptake across the outer membrane into the periplasmic space.

The iron(III) dicitrate transport system of *E. coli* is induced by citrate and low amounts of iron (Hussein et al. 1981). Induction takes place at the level of transcription (Zimmermann et al. 1984). However, the inducers do not have to enter the cytoplasm. Mutants in one of the genes known to encode proteins for iron(III) dicitrate transport across the cytoplasmic membrane are still inducible. Induction is exerted from the periplasmic space across the cytoplasmic membrane with the help of two proteins, one localized in the periplasmic space (FecR) and one in the cytoplasmic membrane (FecI). The membrane-bound protein exhibits a DNA binding motif indicating a transmembrane signal transduction by the same protein which acts as a transcriptional activator. The periplasmic protein is required for iron citrate induction. Uptake of the inducer into the periplasmic space requires the FecA outer-membrane receptor protein and the TonB protein, indicating the involvement of TonB in the iron(III) dicitrate transport across the outer membrane (Van hove et al. 1990).

Recently, cephalosporin derivatives, carrying catechol substituents, were described which exhibited a 100-fold higher antibiotic activity than classical cepha-

losporins against *E. coli* and other Gram-negative bacteria (Watanabe et al. 1987). They showed no higher activity at the target site but an enhanced uptake via a receptor (Fiu or Cir outer-membrane protein, Table 1), and a TonB-dependent transport across the outer membrane (Curtis et al. 1988). Thus, elucidation of the cause of the very low minimal inhibitory concentration of these derivatives provided compelling evidence for a specific outer-membrane transport pathway since these compounds have only to reach the outer surface of the cytoplasmic membrane where their targets, murein biosynthetic enzymes, are located. Furthermore, they demonstrated the chemical nature of iron(III) siderophores taken up via the Cir and Fiu receptors. Synthesis of both proteins is strongly enhanced at iron-limiting growth conditions, and their molecular mass is in the same range as that of the other iron(III) siderophore receptors, from which it was inferred that Fiu and Cir are involved in iron(III) siderophore uptake. Recognition of catechol-type siderophores by Fiu and Cir is supported by the finding that uptake of iron(III) dihydroxybenzoyl-serine occurs via FepA, Cir and Fiu and is abolished in strains mutated in all three genes (Hantke 1990).

A rifamycin derivative (CEP 4832) with a 200-fold higher activity than rifamycin against *E. coli* provides another example of a TonB-dependent outer membrane transport. This derivative is taken up via FhuA and TonB but does not use the FhuBCD proteins to cross the cytoplasmic membrane (Pugsley et al. 1987). The minimal inhibitory concentration for inhibiting the RNA polymerase is the same as with rifamycin so that effective transport across the outer membrane is the cause of the enhanced antibiotic activity of the derivative.

Interaction of the TonB protein with the FhuA protein

Functional restoration of mutated FhuA proteins by mutated TonB proteins

The TonB protein was identified and localized in the cytoplasmic membrane fraction of *E. coli* (Plastow and Holland 1979). In spheroplasts TonB is degraded by added proteinase K (Postle and Skare 1988). Exposure of TonB to proteases in spheroplasts was confirmed by degradation with trypsin (Fischer et al. 1989). However, TonB contains no signal sequence which is cleaved upon translocation of part of the protein across the cytoplasmic membrane. The TonB protein is hydrophilic and contains at the N- and C-terminal only short hydrophobic segments which could serve as cytoplasmic membrane (or outer membrane) anchors.

To examine the role of the hydrophobic sequences, the first 20 N-terminal amino acids were replaced by 8 amino acids of β-galactosidase (T M I T P S L H). Most of the derivative was found in spheroplasts and was degraded by trypsin, indicating a membrane-bound location which was accessible to the protease, probably at the outside of the cytoplasmic membrane. However, a fraction was released upon converison of cells to spheroplasts suggesting a periplasmic location. It seems that the N-terminus is involved in TonB-anchoring in the cytoplasmic membrane, and that the shorter β-galactosidase peptide cannot substitute for the authentic TonB N-terminus. The derivative also showed no TonB activity. A C-terminally modified TonB derivative, in which 38 amino acids were replaced by 4 vector amino acids (Q A Y R), was not released upon conversion of cells to spheroplasts and was degraded by trypsin, indicating anchoring in but exposure at the periplasmic face of the cytoplasmic membrane. Apparently, the hydrophobic segment close to the C-terminus is not required for membrane anchoring.

Location in the cytoplasmic membrane with the extension of part of the protein into the periplasmic space would be an appropriate arrangement for TonB to regulate outer membrane receptor activity in response to the energization of the cytoplasmic membrane. The proposed model predicts two TonB conformations, an energized and an unenergized state, whereby the energized state induces the release conformation of the receptors leading to the transfer of the receptor-bound compounds into the periplasmic space. Induction of the receptor-release conformation consumes energy so that the TonB protein switches to the unenergized conformation and has to be reenergized by the cytoplasmic membrane potential. Such a model implies a physical interaction between the TonB protein and the receptors.

For the interaction between the TonB protein and the various receptors (Table 1), a common structure in the receptors is expected which is recognized by the TonB protein. Indeed, such a structure, comprising a pentapeptide close to the N-terminus, designated the TonB box, has been found in all receptors whose activity depends on TonB. For example, the FhuA TonB box is composed of Asp-Thr-Ile-The-Val, the FhuE sequence reads Glu-Thr-Val-Ile-Val, the Cir sequence Glu-Thr-Met-Val-Val, the BtuB sequence Asp-Thr-Leu-Val-Val.

It was somewhat surprising to find the same TonB box in colicins which are taken up by a TonB-dependent mechanism (summarized in Roos et al. 1989). This could mean that colicins are released from the receptors by the same mechanism as the other substrates but further transport across the outer membrane requires an additional TonB-dependent step. With the exception of the TonB box, the amino acid sequences of the receptors and colicins display little, if any, similarity.

The selected examples were chosen for a more detailed study using site-directed mutagenesis of the TonB box. The results of our own studies with the FhuA receptor and with colicin B are listed in Table 2. Replacement of the isoleucine residue number 9 in the FhuA protein by proline (I9P) rendered the FhuA protein inactive for the uptake of ferrichrome, of the antibiotic albomycin, and almost inactive for the uptake of colicin M (Schöffler and Braun 1989). The V11D replacement in FhuA conferred to cells full resistance to

Table 2. Functional restoration of *fhuA* and *cba* (colicin B) TonB box mutations by *tonB* mutations

FhuA	Growth on ferrichrome of			
	TonB wild-type	TonB (Q160L)	TonB (Q160K)	TonB (R158L)
Wild-type	+++	+++	+++	+++
I9P	−	−	++	++
D7G+I9N	−	−	++	−
I9T	+++	+++	+++	nel
V11A	++	++	++	(+)
V11D	−	−	−	−
Cba	**Sensitivity of cells**			
Wild-type	+++	+++	+++	+++
V20A	+++	+++	+++	+++
V20G	++	++	++	++
V20R	−	(+)	−	(+)
V20E	−	(+)	−	−
M18S	(+)	(+)	(+)	(+)
M18N	((+))	(+)	−	((+))
M18P	−	−	−	−
D16A	+++	+++	+++	+++

Growth on ferrichrome was tested with filter paper disks impregnated with 10 nmol ferrichrome. The *aroB* mutant used was unable to grow on nutrient broth supplemented with 0.2 mM 2,2′-dipyridyl. The density and the diameter of growth zones around the disks was recorded. −, means no growth stimulation (Schöffler and Braun 1989). Sensitivity to colicin B was determined by dropping 5 µl of 10-fold dilutions onto tryptone/yeast agar plates seeded with 10^8 indicator bacteria. +++, means full sensitivity; ++, a 10-fold and +, 100-fold reduced sensitivity; (+), a turbid and ((+)), a very turbid zone of growth inhibition. A more detailed account of the results and the methods used is contained in Mende and Braun (1990). The *fhuA, cba* and *tonB* genes to be tested were on plasmids in strains which carried on the chromosome no active *fhuA, cba* or *tonB* gene. Nel, not determined

albomycin and colicin M and no growth on ferrichrome as the sole iron source.

If the defects caused by these FhuA mutants were due to an inappropriate physical interaction with the TonB protein, altered TonB protein derivatives could be expected which once again interacted with the altered FhuA protein (Fig. 1). Indeed, such TonB derivatives were found. They were originally isolated using the *btuB451* mutation which conferred normal binding but no uptake of vitamin B_{12} (Heller et al. 1988). The

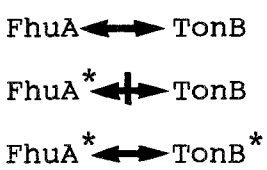

Fig. 1. Proposed interaction between the FhuA and the TonB protein which is abolished by point mutations in the TonB box of FhuA (for example isoleucine→proline at residue 9 of the mature protein), and restored by an additional point mutation at residue 160 (glutamine→lysine), or 158 (arginine→leucine) of the TonB amino acid sequence. The stars indicate the mutated proteins and the double arrow interaction (interrupted arrow lack of interaction) between the proteins

point mutation in the TonB box caused the replacement of leucine by proline at position 8 of the mature protein (L8P). Of 10 independently isolated *tonB* suppressor mutants restoring growth of the *btuB451* mutant on 5 nM vitamin B_{12}, all were affected in the same *tonB* codon, number 165 of the open reading frame (Postle and Good 1983) which is actually number 160 of the translation product (Postle and Skare 1988). Three of the mutations gave rise to a Gln→Lys replacement, and seven to a Gln→Leu substitution. One *tonB* mutation (Q160K) which suppressed the BtuB (L8P) mutation also suppressed the FhuA (I9P) mutation (Table 2), which strongly indicates that the mutated sites on the receptors are functionally equivalent, and that the mutated site on the TonB protein defines a locus which is involved in the interaction with both receptors. Subsequent studies led to only one additional *tonB* suppressor mutation in the same codon (Q160P) restoring uptake of vitamin B_{12} into a BtuB (V10G) mutant (Bell et al. 1990), and one mutation (*tonB* R158L) which suppressed the *fhuA* (I9P) mutation (Table 2).

The TonB box residue of colicin B equivalent to FhuA (I9) and BtuB (L8) is methionine number 18. When this was replaced by proline, serine, or asparagine colicin B uptake, but not colicin B binding to the receptor, was strongly impaired (Table 2) (Mende and Braun 1990). The same was true when the valine residue 20, which is conserved in all TonB box sequences, is substituted by ariginine or glutamate. The colicin B derivative (M18P) was still active and killed cells when the outer membrane permeability barrier was overcome by osmotic shock treatment. It also formed pores in black lipid membranes displaying the same single-channel conductance as wild-type colicin B (Mende and Braun 1990). Therefore, inactivity against whole cells must come from the lack of uptake. Some of the *tonB* mutations suppressing certain *fhuA* mutations, also suppressed some of the colicin B TonB box mutations (for example V20R, V20E). These results demonstrate that the TonB box of colicin B has the same functional properties as the TonB box of the receptor proteins. Allele-specific suppression of a mutation in one gene by mutation in another gene strongly suggests a direct physical as well as functional interaction between the two gene products.

Physical stabilization of the TonB protein by the FhuA protein

The TonB protein is functionally (Bassford and Kadner 1977) and physically (Postle and Skare 1988) unstable. The half-life of the protein at 42° C is 10 min. We took advantage of this fact to examine whether physical interaction between the TonB protein and the FhuA protein could be demonstrated by a delay in TonB degradation. Since the amount of chromosomally expressed TonB protein is too low to be unambiguously identified by autoradiography on polyacrylamide gels, the *tonB* gene was cloned downstream of the strong gene 10 promoter of phage T7. Exclusive *tonB* transcription was

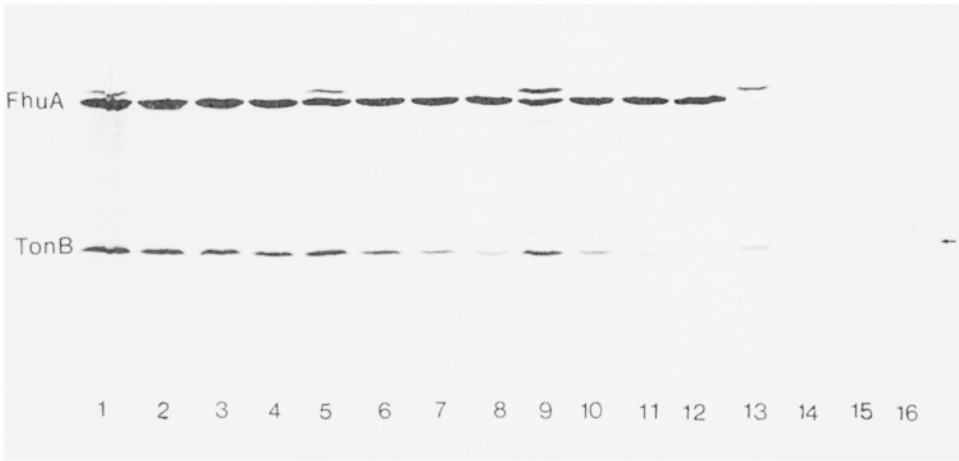

Fig. 2. Stabilization of the mutated TonB protein (Q160L) by the wild-type FhuA protein (lanes 1–4), and weak or no stabilization by the derivatives FhuA (I9P; lanes 5–8), FhuA (V11D; lanes 9–12), and FhuA (V11A; lanes 13–16). The mutated *tonB* gene, the wild-type *fhuA* gene and the mutated *fhuA* genes were cloned on the same plasmid pT7-5 and expressed in strain WM1576 pGP1-2 (Tabor and Richardson 1985). Cells in the logarithmic growth phase were harvested, suspended in methionine assay medium, shaken at 27° C for 1 h, and then shifted for 15 min to 42° C for induction of phage T7 polymerase synthesis. Rifamycin (0.2 mg/ml) was added and incubation continued for a further 15 min at 42° C followed by 30 min at 27° C. Cells were then labeled at 30° C for 5 min with [^{35}S]methionine (740 kBq), followed by a chase with nonradioactive methionine (50 mM). Samples were withdrawn after 0 (lanes 1, 5, 9, 13), 30 min (lanes 2, 6, 10, 14), 60 min (lanes 3, 7, 11, 15) and 90 min (lane 4, 8, 12, 16) of the chase period, and subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis and autoradiography as described (Fischer et al. 1989; Günter and Braun 1990). The band above the FhuA protein is the biosynthetic FhuA precursor containing a signal peptide which is most pronounced in the zero time chase samples. The arrow at the right indicates the barely detectable residual TonB protein

achieved by the T7 RNA polymerase which recognizes only T7 promoters, and by selective inhibition of the *E. coli* RNA polymerase with rifamycin. No protection against degradation was observed with FhuA synthesized from the chromosomal *fhuA* gene. However, increased amounts of FhuA cloned on the same vector as the *tonB* gene prevented degradation of the TonB protein.

In Fig. 2, a series of experiments is shown with TonB (Q160L) combined with wild-type FhuA (lanes 1–4), FhuA (I9P; lanes 5–8), FhuA (V11D; lanes 9–12), and FhuA (V11A; lanes 13–16). The degree of stabilization can be seen by comparison of wild-type FhuA, which functionally interacts with TonB (Q160L), and the FhuA derivatives with low (I9P) or no (V11D) functional TonB coupling. Functional interaction between FhuA (I9P) and TonB (Q160L) is not strong enough to restore growth on ferrichrome but colicin M sensitivity is tenfold higher compared with cells carrying FhuA (I9P) and TonB wild-type. A much lower stabilization occurs by FhuA (V11D) for which no functional activity was recorded (Table 2). Interestingly, only a small fraction of the FhuA (V11A) protein is exported and is degraded. The small amount of processed and unprocessed FhuA protein stabilizes TonB (Q160L) only very weakly. Functional activity (with regard to FhuA and TonB) was observed in the very sensitive assay for which much fewer protein molecules are required than for the labeling experiment. All 16 possible combinations between wild-type TonB, the 3 TonB derivatives, and wild-type FhuA plus the 3 FhuA derivatives were tested by pulse/chase experiments; the result was that TonB degradation was delayed when a functional interaction occurred (growth promotion by ferrichrome) but much less protection occurred if no growth on ferrichrome was demonstrated (Günter and Braun 1990). We take these results as evidence for a physical interaction between the FhuA and TonB proteins and conclude that this interaction forms the basis of their concerted activity in ferrichrome, albomycin and colicin M uptake and for sensitivity to the phages T1 and Φ80.

The ExbBD and TolQRA proteins for outer-membrane transport

Originally the term *exb* defined mutations which conferred insensitivity to colicin B through a compound which was excreted into the culture medium. This compound was later identified as enterochelin (enterobactin), which was synthesized in large quantities due to an iron deficiency caused by the *exb* mutation. Iron(III) enterochelin competes for the common receptor protein FepA of colicin B. The *exbA* gene was found to be the *tonB* gene, while *exbB* defined a new locus at min 65 of the 100-min *E. coli* linkage map. Mutants in *exb* are impaired in iron transport via enterochelin and ferrichrome.

DNA of the *exb* locus has been cloned and sequenced. It consists of two open reading frames, termed *exbB* and *exbD*, which are both required to complement *exb* mutants (Eick-Helmerich and Braun 1989). Surprisingly, the amino acid sequence derived from the *exbB* nucleotide sequence showed 26% iden-

Table 3. Properties of *exbB* and *tol* mutants

Strain	Colicins						Phages	
	B	D	M	E1	E2	K	T1	Φ80
GM wild type	2(4)	3(5)	3(5)	2(3)	3(5)	2	6	7
TPS13 *tolQ*	2(4)	3(5)	3(5)	r	(2)	r	6	7
HE2 *tolQ exbB*	r	r	r	r	r	r	r	r
HE5 *tolA*	2(3)	3(4)	3(4)	r	r	r	6	7
HE7 *tolA exbB*	2(3)	2(3)	3(4)	2(3)	2(3)	1(2)	6	7
HE6 *tolB*	2(3)	3(4)	3(4)	2(3)	r	r	6	7
HE8 *tolB exbB*	1(2)	2(3)	2(3)	2(3)	r	(2)	6	7
2559 *tolR*	2(4)	3(5)	3(5)	r	r	r	6	7
HE10 *tolR exbB*	r	r	r	r	r	r	r	r
HE1 *exbB*	(1)	(2)	(3)	2(3)	3(5)	2	6	7

The last of 10-fold dilutions which resulted in a clear (for number in brackets, turbid) zone of growth inhibition are listed. For example, 2 indicates that the colicin solution could be diluted 10^2-fold to yield a clear zone on a lawn of bacteria seeded on tryptone/yeast agar plates (for experimental details see Eick-Helmerich and Braun 1989). The strains, except for 2259 and HE10, are described in Braun 1989. r, resistant to undiluted solutions

tity and 79% similarity with the TolQ sequence, and ExbD was 25% identical and 70% similar to the TolR sequence. The *tol* locus consists of four genes, designated *tolQRAB*, in which mutations confer insensitivity to colicins A, E, K, L, and N (Sun and Webster 1987). These colicins, in contrast to the colicins listed in Table 1, do not require the TonB protein to enter cells.

The structural similarity between the ExbBD proteins and the TolQR proteins suggests a common ancestor. During evolution, the original genes may have been duplicated and developed independently. Both groups of genes then maintained their original function in the specific uptake of certain biopolymers, for which biomembranes are otherwise impermeable. However, the ExbBD and TolQR proteins are each specialized for the exclusive uptake of only one group of colicins. Colicins also seem to have evolved from one or a few ancestral genes (Ross et al. 1989) and their diversity may have developed on a similar time scale as the ExbBD and TolQR proteins.

The hypothesis of a functional connection between the Exb and Tol proteins gained support by the finding that the TonB-dependent sensitivity to colicins, which is strongly reduced but not fully absolished by mutations in the *exbBD* locus, was completely lost by an additional mutation in the *tolQ* gene (Braun 1989). Double mutants in *exbBD tolQ* were completely insensitive to colicins B, D, M and to phages T1 and Φ80 (Table 3). Resistance to the phages was particularly striking since single mutants in either *exb* or *tol* were nearly fully sensitive (Braun 1989). Complete phage T1 and Φ80 resistance was also determined with *exbBD tolR* double mutants (Table 3). Insensitivity concerns colicins with a TonB-dependent and a TonB-independent uptake mechanism (Table 3). Although *tolQ* and *tolR* single mutants have been listed as being resistant to colicins E1, E2, and K, one can frequently observe turbid zones of growth inhibition with undiluted solutions which never appear in *tolQ exbB* and *tolR exbB* double mutants, indicating partial substitution of the missing *tol* gene function by the *exbB* gene function.

The double mutant HE7 *tolA exbB* showed an interesting phenotype in that loss of sensitivity in single mutants (*exbB* for colicins B, D, M; *tolA* for colicins E1, E2, K) was reversed in the double mutant (Table 3). No counterpart for the *tolA* gene is known in the *exb* locus so that the mutual compensation of a single defect by an additional defect has no explanation. Restoration of activity was also observed for the uptake of ferrichrome. The *exbB* mutant H1843, which was unable to synthesize its own siderophore enterochelin due to an *aroB* mutation, could not grow on ferrichrome added to iron-depleted nutrient broth/dipyridyl medium. Growth was restored by introducing the *tolA* mutation of strain A592. The same was observed with citrate as the siderophore, although less pronounced, since iron(III) dicitrate is taken up into *exbB* mutants, but with a slower rate (Hantke and Zimmermann 1981).

Stabilization of the TonB protein by the ExbB protein and TolQR proteins

A physical interaction between the ExbBD and TolQR proteins with the TonB protein was examined as has been described above for the TonB stabilization by the FhuA protein. Since none of the presumed reaction partners can be identified by autoradiography on polyacrylamide gels without selective overexpression, the *exbBD* genes were cloned separately and together on the same plasmid as the *tonB* gene under the control of the phage T7 gene 10 promoter (Fischer et al. 1989). In addition, the *tolQR* genes were cloned downstream of the *tonB* gene (C. Herrmann, this institute). Degradation of the TonB protein was prevented by the ExbBD and the TolQR proteins (Fig. 3). With the separately cloned *exbB* and *exbD* genes it became apparent that ExbB stabilized TonB, but ExbD had no stabilizing effect on TonB (Fischer et al. 1989), demonstrating the specificity of the interaction. We take the stabilization as evidence for a mechanical interaction between the TonB protein and the ExbB/TolQ proteins. Further-

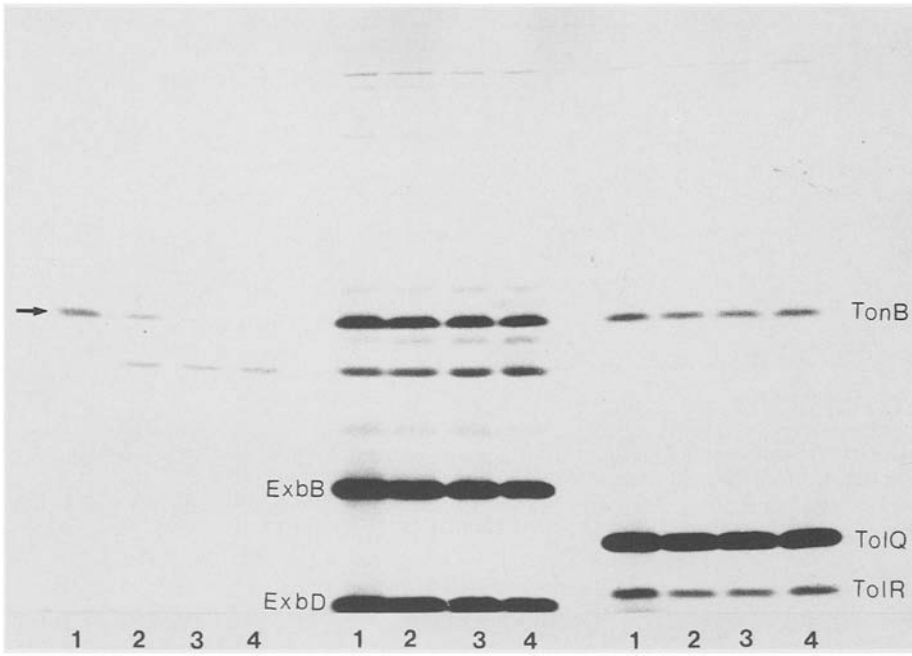

Fig. 3. Stabilization of the TonB protein by the ExbB and TolQR proteins. The pulse/chase experiment was performed as described in Fig. 2. Plasmid pCG754 carries the *exbB, exbD* and *tonB* genes downstream of the phage T7 gene 10 promoter (Fischer et al. 1989). The *tolQR* genes were subcloned from plasmid pHH5 which carried a *Sau*3A chromosomal DNA fragment of *E. coli* AB2847 in the *Bam*HI site of pACYC184. Plasmid pHH5 complemented *E. coli* TPS13 *tolQ*, H2259 *tolR* and A592 *tolA*, but not A593 *tolB*, so that it carried the *tolQRA* genes. A DNA fragment bearing the *tolQR* genes were excised with *Hind*III/*Sal*I from pHH5 and cloned into pCG752 cleaved with *Hind*III/*Sal*I. pCG752 carries the *tonB* gene under phage T7 gene 10 control (Fischer et al. 1989). The restriction sites were taken from the published *tolQR* sequence (Sun and Webster 1987). The resulting plasmid pHE10 conferred strain HE2 *exbB tolQ* sensitivity to phage Φ80, albomycin and colicins B and M. *E. coli* WM1576 pGP1-2 carrying in addition pCG752 $tonB^+$ (left panel), pCG754 $tonB^+$ $exbB^+$ $exbD^+$ (center panel), and pHE10 $tonB^+$ $tolQ^+$ $tolR^+$ (right panel) was pulse-labeled for 5 min with [^{35}S]methionine and then chased with a surplus of unlabeled methionine for 0, 5, 15, and 60 min (lanes 1–4). The arrow indicates the position of the labeled TonB protein on the autoradiograph

more, ExbB and ExbD bind to each other since ExbB prevents degradation of ExbD by cellular proteases and by added trypsin and proteinanse K (Fischer et al. 1989).

Conclusions

Our approach to studying outer and inner membrane coupling has provided valuable insights into a conceptually novel and technically difficult system. Processes occurring in two membranes have to be studied simultaneously. Metabolism in the cytoplasmic membrane directly activates proteins in the outher membrane. Isolation of the interacting proteins in a pure, native state to examine their interaction would be a difficult task due to their very low amounts. Moreover, the proposed change in conformation as a response to membrane energization cannot be studied with the isolated proteins. The TonB protein and the ExbBD and TolQR proteins have mainly been localized in the cytoplasmic membrane, although portions have also been found in the outer membrane fraction (Bourdineaud et al. 1989; Eick-Helmerich and Braun 1989; see also discussion in Postle and Skare 1988). It is not clear whether these findings reflect the true subcellular distribution, or whether they are partial artefacts due to overexpression of these proteins required for their identification. An artractive hypothesis places these proteins into adhesion zones between the outer and the cytoplasmic membrane where entry of colicins and of phage DNA could also take place.

Stabilization of the TonB protein by the FhuA protein, by the ExbB protein and the TolQR proteins indicates a physical interaction between these proteins. Such an interaction is the prerequisite for activation of the FhuA protein by the TonB protein. TonB activity also depends on the ExbBD proteins, and their function can be partially replaced by the TolQR proteins. Protection against degradation by cellular proteases does not necessarily imply that TonB stabilization is the way these proteins affect TonB activity. Regulation of TonB activity could be much more subtle. For example, TonB may not sense directly the energized state of the cytoplasmic membrane but rather require the ExbBD and/or TolQR proteins.

We have relied on overexpression of the proteins for their identification. The genes were cloned on the same plasmid and transcribed under the control of the same promoter. Stabilization was only observed when the proteins were overexpressed, suggesting a stoichiometric rather than catalytic interaction. Overexpression may affect the subcellular localization and the functional properties, but stabilization would not occur

without an affinity between these proteins. The specificity of interaction was demonstrated by the preferential stabilization of TonB/FhuA pairs which interact functionally, TonB and ExbD stabilization by ExbB, and the lack of TonB stabilization by the ExbD protein.

Regarding iron transport, we visualize the following scenario (Fig. 4). Transport across the outer membrane occurs independent of the transport across the cytoplasmic membrane. Ferrichrome binds to the FhuA receptor protein at the cell surface. In this way the iron(III) siderophore is extracted from the culture medium and concentrated at the cell surface. Translocation across the outer membrane requires cellular energy and the TonB protein. The TonB protein binds to the FhuA protein and induces the release conformation on FhuA. Ferrichrome enters the periplasmic space where it binds to the FhuD protein (no data are available which support or discount the involvement of FhuD in the release of ferrichrome from FhuA). The FhuD protein, in turn, donates ferrichrome to the FhuB protein which, in an energy-dependent process, translocates ferrichrome across the cytoplasmic membrane. For this step energy is provided through the FhuC protein which contains sequences typical for ATP binding proteins. As inferred from other binding-protein-dependent transport systems, it is proposed that ATP hydrolysis provides the energy. Inside the cytoplasm, iron(III) is released from ferrichrome by reduction to Fe(II), for which a soluble *E. coli* reductase has been purified to homogeneity, and a membrane-bound reductase activity has been identified (Fischer et al. 1989). Deferri-ferrichrome is then inactivated by acetylation at one of the *N*-hydroxy groups and excreted into the culture medium (Hartmann and Braun 1980; Schneider et al. 1981).

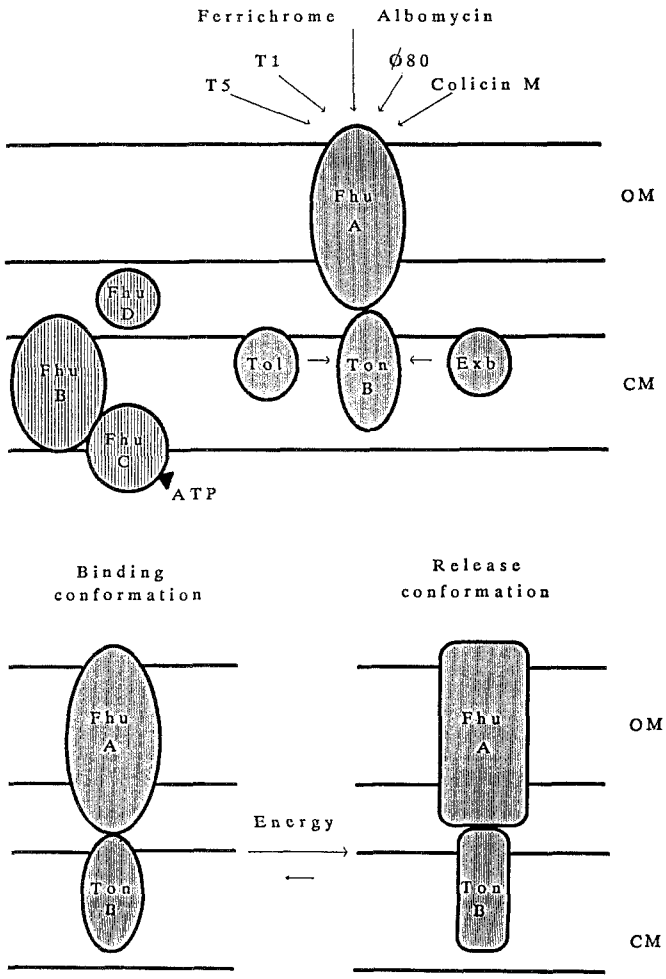

Fig. 4. Model describing our current view of iron uptake into *E. coli*. The multifunctional FhuA receptor binds ferrichrome and analogs such as ferrichrysin, ferricrocin and albomycin, the phages T5 (TonB-independent), T1 and *Φ*80, and colicin M. Translocation is achieved by a conformational change of the FhuA protein induced by the TonB protein and presumably by the cytoplasmic membrane potential. It is proposed that TonB fluctuates between an energized and a nonenergized conformation whereby the energized state induces the release conformation in the FhuA protein. Transport across the cytoplasmic membrane is TonB-independent and catalyzed by the FhuBCD proteins. OM, outer membrane; CM, cytoplasmic membrane. ATP indicates a potential binding site of ATP at the FhuC protein which contains typical nucleotide binding domains

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