

The Structurally Related *exbB* and *tolQ* Genes Are Interchangeable in Conferring *tonB*-Dependent Colicin, Bacteriophage, and Albomycin Sensitivity†

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Double *exbB tolQ* mutants of *Escherichia coli* were completely resistant to bacteriophages T1 and ϕ 80, in contrast to strains with *exbB* or *tolQ* mutations, which were sensitive. Cells carrying mutations in *exbB* were partially tolerant to colicins B, D, and M and became fully tolerant by the introduction of *tolQ* mutations. This suggested involvement of both *exbB* and *tolQ* in *tonB*-dependent uptake.

Escherichia coli excludes biopolymers, with the exception of bacteriophage DNA and colicins, which are taken up very efficiently. Mutations conferring resistance to several colicins and phages are likely to be in genes which determine general uptake routes for biopolymers. In contrast, mutations in genes encoding cell surface receptors usually affect the uptake of a single compound or only a few compounds. The *tonB*, *exb*, and *tol* loci encode functions which are required for the uptake of a variety of biopolymers which use different receptors and therefore may define more general uptake routes.

Cells carrying mutations in the *tonB* gene are tolerant to colicins B, D, G, H, I, M, and V (group B colicins) (3, 15) and to phages T1 and ϕ 80 (8). The colicins adsorb to their receptors at the cell surface but are not internalized by *tonB* mutants (2). The phages only adsorb reversibly to *tonB* mutants. Irreversible adsorption, accompanied by DNA release, only occurs in energized *tonB* wild-type cells (8). T1 host range mutants were isolated which infected *tonB* mutants. They were inactivated by FhuA receptor-containing outer membranes, in contrast to wild-type T1, which remained active (9). Apparently, irreversible binding of the phages and translocation of the colicins across the outer membrane require a certain conformation of the receptor proteins which is induced by energy mediated via the TonB protein. Uptake of the colicins, except colicin V, also depends on *exb* (3, 15), which was recently shown to consist of two genes, termed *exbB* and *exbD* (5). While *tonB* mutants are tolerant to the highest activities of colicin solutions available (dilution titers, 10^5), *exb* mutants only show a reduced sensitivity which, however, can amount to several orders of magnitude, depending on the colicin tested (5).

Cells carrying mutations in the *tol* locus near *gal* at 16.5 min of the *E. coli* linkage map are tolerant to colicins A, E1, E2, E3, K, L, N, and S4 (group A colicins) (1, 4, 12, 17). Various combinations of tolerance to these colicins were classified as TolI to TolXVII. Recently, four genes were identified in the *tol* locus and designated *tolQ*, *tolR*, *tolA*, and *tolB* (18). Strains carrying mutations in *tolQ*, *tolR*, or *tolA* were tolerant to colicins E1, E2, and E3 (the other colicins were not mentioned) and to phages f1 and IKE, whereas *tolB* mutants were sensitive to these filamentous phages and to colicin E1 (18).

Regarding the uptake routes, the group A colicins (*tonB*-

independent uptake) and the filamentous phages form one class which is separate from the group B colicins and the phages T1 and ϕ 80. Cross-tolerance occurs within the classes but not between the classes. When we noticed the 26% identity (additional 79% similarity) in the amino acid sequences of the ExbB and TolQ proteins and the 25% identity (70% similarity) between the ExbD and TolR proteins (5), the question arose whether functional relationships between the Exb and Tol proteins still exist or whether the divergent evolution had lasted long enough to extinguish indications for a once common function of the proposed ancestor of the Exb- and Tol-related uptake routes (5).

The wild-type *tonB⁺ exb⁺ tol⁺* strain GM1 (Table 1) was sensitive to colicins B, D, and M, to phages T1 and ϕ 80, and to the antibiotic albomycin (Table 2). The latter was included in this study since it is taken up via FhuA, TonB, and Exb (6, 9, 10), as it is too large (molecular mass, 1,045 daltons) for passive diffusion through the porins. Strain HE1, derived from GM1 by P1 transduction of *exbB::Tn10* of strain H1388 (5), was tolerant to colicin B and albomycin, while colicins D and M gave rise to turbid zones of growth inhibition (Table 2). Sensitivity to phages T1 and ϕ 80 remained unaltered. The *tolQ* derivative was as sensitive as GM1 to colicins B and D, the phages, and albomycin and was 10 times less sensitive to colicin M. However, the *exbB tolQ* double mutant HE2 was completely tolerant to all the compounds at the highest concentrations available (Table 2). This shows for the first time that *tolQ* contributes to the sensitivity of cells to group B colicins and to phages T1 and ϕ 80. Sensitivity of HE2 to all compounds was completely restored by transformation with plasmid pKE7 carrying *exbB* and *exbD* on pUC18 (5). Transformants carrying pKE70 with *exbB* on pDS6 (5) exhibited a strongly reduced sensitivity to the colicins and to albomycin, phage T1 formed turbid plaques when the original suspension was 10^7 -fold diluted, and sensitivity to phage ϕ 80 was 10-fold reduced. Interestingly, lack of *exbB*- and *tolQ*-encoded functions was overcome by overproduction of the TonB protein required for T1 sensitivity (Table 2). This shows that TonB alone, in the absence of ExbB and TolQ, is able to specifically confer sensitivity to T1. In contrast, phage ϕ 80 formed only turbid plaques up to a dilution of 10^{-3} . Tolerance to the colicins and albomycin was unaltered. Sensitivity to all agents was largely restored in transformants of HE2 bearing the *tonB*, *exbB*, and *exbD* genes on pCG754 (7). The strongly reduced sensitivity to albomycin

† Dedicated to Hans Zähler on the occasion of his 60th birthday.

TABLE 1. *E. coli* strains and plasmids used

Strain or plasmid	Genotype	Source or reference
Strains		
AB2847	<i>aroB thi malT tsx</i>	This institute
BR158	AB2847 <i>tonB</i>	This institute
H1388	<i>aroB pro lac malT tsx thi exbB::Tn10</i>	5
W3110	Wild type	This institute
W3110-6	W3110 <i>exb</i>	5
H1443	<i>araD Δlac aroB rpsL</i>	K. Hantke
H1843	H1443 <i>exbB::Mu d1</i>	K. Hantke
GM1	<i>ara Δ(lac pro) thi F' lac pro</i>	16
TPS13	GM1 <i>tolQ</i>	17
A592	<i>tolA1 fhuA21 lacY1 thi leuB6 supE44 thr-1</i>	B. J. Bachmann
A593	<i>tolB1 fhuA21 lacY1 thi leuB6 supE44 thr-1</i>	B. J. Bachmann
HE1	GM1 <i>exbB::Tn10</i>	This study
HE2	TPS13 <i>exbB::Tn10</i>	This study
HE5	A592 <i>fhuA</i> ⁺	This study
HE6	A593 <i>fhuA</i> ⁺	This study
HE7	HE5 <i>exbB::Tn10</i>	This study
HE8	HE6 <i>exbB::Tn10</i>	This study
Plasmids		
pCG752	pT7-5 carrying <i>tonB</i>	7
pKE7	pUC18 carrying <i>exbB exbD</i> Ap ^r	5
pKE70	DS6 carrying <i>exbB</i>	5
pCG754	pT7-5 carrying <i>tonB exbB exbD</i>	7
pHE10	pHSG575 carrying <i>fhuA</i>	K. Hantke
pHE11	pHSG575 carrying <i>fhuA</i>	K. Hantke

was previously observed with transformants carrying multi-copy plasmids bearing various uptake genes (7).

Strains A592 (*tolA*) and A593 (*tolB*) were sensitive to colicins B and D (data not shown). The other compounds could not be tested because of *fhuA*-conferred resistance.

TABLE 2. Comparison of cell sensitivities to phages, colicins, and albomycin

Strain (relevant genotype or plasmid)	Endpoint dilution of ^a :					
	Colicin			Phage		Albo- mycin
	B ^b	D ^b	M ^b	T1 ^b	φ80 ^b	
GM1	2 (4)	3 (5)	4 (6)	7	7	2 (4)
HE1 (<i>exbB</i>)	(2)	(2)	(3)	7	7	r
TPS13 (<i>tolQ</i>)	2 (4)	3 (5)	3 (5)	7	7	2 (4)
HE2 (<i>exbB tolQ</i>)	r	r	r	r	r	r
HE2 (pKE7)	2 (4)	3 (5)	4 (6)	7	7	2 (4)
HE2 (pKE70)	(3)	(4)	(4)	(7)	6	2 (3)
HE2 (pCG752)	r	r	(2)	7	(3)	r
HE2 (pCG754)	2 (3)	3 (4)	4 (5)	7	6	(3)
HE5 (<i>tolA</i>)	2 (3)	3 (4)	3 (4)	7	7	c
HE7 (<i>tolA exbB</i>)	1 (3)	1 (3)	3 (4)	7	7	c
HE6 (<i>tolB</i>)	2 (3)	3 (4)	4 (5)	7	7	c
HE8 (<i>tolB exbB</i>)	1 (3)	2 (3)	3 (4)	7	7	c

^a The last of 10-fold dilutions which resulted in a clear (for numbers in parentheses, turbid) zone of growth inhibition are listed. For example, 7 indicates that the phage suspension could be diluted 10⁷-fold to yield clear plaques. r, Resistant to undiluted solutions.

^b Compounds (3 μl) were spotted on tryptone-yeast agar plates seeded with 10⁸ cells.

^c The *fhuA* mutation had a polar effect on the expression of downstream *fhuCDB* genes so that albomycin was not taken up.

Therefore, pSH575, a low-copy plasmid carrying the *fhuA* gene, was transformed into strains A592 and A593 (Table 1). The resulting strains, HE5 and HE6, were almost fully sensitive to colicin M and to the phages T1 and φ80. The *exbB* derivatives HE7 and HE8 exhibited a slightly reduced sensitivity to the colicins but were fully sensitive to the phages (Table 2). Sensitivity to albomycin could not be tested because the *fhuA* mutation apparently extended into the nearby *fhuCDB* genes, which are required for the uptake of albomycin across the cytoplasmic membrane. It is concluded that the *tolA* and *tolB* gene products are not important for the *tonB*-dependent uptake processes measured in this paper.

To make sure that the results obtained were independent of the strains used and were confined to the *tol* and *exb* loci, additional *tol* and *exb* mutants with a different genetic background were isolated. First, a *nadA::Tn10* mutation was placed by phage P1 transduction close to the *tol* locus of strains A592, A593, and TPS13, followed by cotransduction of Tn10 with either *tolA*, *tolB*, or *tolQ* into strain H1443. The *tol* mutants were sensitive to 2.5% cholerae (1, 4, 12, 17). By the same procedure, *tolA*, *tolB*, and *tolQ* mutants of strain H1843 *exbB* were constructed. The phenotypes of the *tol* mutants and of the *tol exbB* mutants obtained agreed with the properties of the mutants listed in Table 2. In addition, a DNA fragment of strain AB2847 cloned into plasmid pACYC184 complemented TPS13 and A592, but not A593, to *tol*⁺, and restored the T1 sensitivity of strain HE2, showing that the DNA fragment carried the *tolQA* genes (and probably *tolR* located between *tolQ* and *tolA* [18]) and that the *tolQ* mutation combined with the *exbB* mutation conferred T1 resistance.

The data presented in this and previous reports (5, 7) support a model in which the ExbB and ExbD proteins are required for TonB activity. The ExbB and ExbD functions can partially be replaced by the TolQ and TolA functions and presumably by the TolR function, for which no mutant was available. The leaky phenotype of *exb* mutants observed in previous studies came from the functional *tol* genes. Only *exbB tolQ* double mutants were fully resistant to the colicins and the phages. Resistance was as tight as that in *tonB* mutants. This was most obvious with phage sensitivity, for which only the combination of both mutations led to complete resistance, in contrast to the single mutations, for which no phenotype has hitherto been reported. The spot test used in this study also revealed no reduced phage sensitivity of *exbB* and *tolQ* mutants when compared with their parent strains. A more sensitive method was determining the number of plaques formed by phages T1 and φ80 when spread on agar plates seeded with 10⁸ cells. Three pairs of *exbB* wild-type and *exbB* mutant strains were used to avoid influences from strain-specific peculiarities. The plating efficiency of T1 was 61% on HE1 (compared with its efficiency on GM1), 76% on H1388-AB2847, and 60% on W3110-6-W3110. The corresponding figures for phage φ80 were 31, 31, and 9%, respectively. Apparently, mutations in *exbB* reduced the plating efficiency of both phages and more so for phage φ80 than for phage T1. The stricter dependence of φ80 on *exbB* is also obvious from the data presented in Table 2. In contrast, strain TPS13 *tolQ* was at least as sensitive to T1 and φ80 as GM1 (in some experiments, 10 to 30% more plaques were counted on TPS13). The finding that *exbB* and *tolQ* single mutations have only minor or no effects on T1 and φ80 sensitivities supports the conclusion that the functions of both loci are interchangeable for T1 and φ80 infections.

If ExbB and TolQ are part of a complex that activates TonB, then host range mutants of T1 which infect *tonB* mutants should be able to infect *exbB tolQ* mutants and vice versa. Therefore, we isolated as previously described (9) host range mutants of T1 by using *E. coli* BR158 *tonB* (designated T1h1) and HE2 (designated T1h2). A total of 313 plaques were obtained from T1h1 on AB2847, the *tonB*⁺ parent of BR158, 361 were obtained on BR158, 191 were obtained on TPS13, and 164 were obtained on HE2. T1h2 gave rise to 387 plaques on AB2847, 411 on BR158, 161 on TPS13, and 153 on HE2. This shows that T1 host range mutants can be obtained which multiply on the *exbB tolQ* double mutant and on the *tonB* mutant as well. The lower plating efficiency on TPS13 and HE2, compared with that on AB3847 and BR158, is a property of the parent strain GM1, on which, for example, T1 wild type plated 49% less efficiently when compared with AB2847.

Since bypass of TonB also bypassed ExbB-TolQ and vice versa, it is concluded that the same step in T1 infection is affected. Most likely, this step is not the uptake of T1 DNA since it is difficult to imagine that a mutation in T1 DNA leads to a different uptake route into the cell. Rather, it is proposed that the T1h mutants bind irreversibly to the FhuA receptor, which is in a conformation not recognized by T1 wild type. The latter requires a FhuA conformation which is created by energized cells and transmitted from the cytoplasmic membrane to the outer membrane receptor via the TonB protein. Evidence for a functional as well as physical coupling between FhuA and TonB was recently obtained by isolating mutations in the TonB box of *fhuA* which were suppressed by mutations in *tonB*. One of the *fhuA* mutants carried a valine-to-aspartate replacement at position 11 of the mature protein which rendered the mutant inactive in *fhuA*-related activities requiring *tonB*. After transformation with a plasmid carrying wild-type *tonB*, cells became sensitive to phage T1 only in the presence of ferrichrome, which uses FhuA as its binding site and TonB and ExbB-ExbD for uptake (16). Apparently, ferrichrome induced a conformation of FhuA to which T1 was able to bind. This shows that the FhuA receptor is subject to conformational changes which are recognized by phage T1. The T1h phages exhibit an extended host range in that they recognize an unenergized as well as an energized FhuA conformation.

Previously, we rendered *tonB* (2) and *exbB-exbD* (5) mutants sensitive to colicin M by osmotic shock treatment. This procedure makes the outer membrane temporarily permeable to proteins (13). Of the colicins employed in this study, colicin B forms small channels in the cytoplasmic membrane (14), colicin D has to enter the cytoplasm as an inhibitor of protein synthesis (19), and colicin M integrates into the cytoplasmic membrane, since it inhibits murein synthesis at the stage of bactoprenol phosphate regeneration (11). Uptake of these colicins into and across the cytoplasmic membrane certainly differs. The uptake route of albomycin through the cytoplasmic membrane is known (6) and has nothing in common with that of the colicins. The common denominator for all these compounds is the dependence on receptors and the *tonB*-, *exbB-exbD*-, and *tolA-tolQ*-encoded functions which strongly supports involvement of all these functions in uptake across the outer membrane via a common mechanism. A model which integrates these observations proposes a protein complex in the cytoplasmic membrane composed of TonB, ExbB-ExbD, and TolA, TolQ and TolR. The ExbB-ExbD proteins activate TonB, and one mode of action seems to be the prevention of TonB degradation by ExbB (5). The Tol proteins also

contribute to the activity of TonB, but independent of TonB they participate in energy-dependent uptake of group A colicins and in infection by filamentous phages. *exbB* also contributed to the *tol*-dependent sensitivity of cells to colicin E2, since only strain HE2 was completely resistant to colicin E2, in contrast to TPS13, on which a turbid growth inhibition zone was formed by a 10³-fold-diluted E2 stock solution, while the same E2 concentration yielded a clear inhibition zone on GM1 and a turbid zone when diluted 10⁵-fold.

The novel finding of this report is the contribution of *tol*- and *exb*-encoded functions to uptake processes which hitherto were considered to be either *tol* or *exb* dependent. This was most obvious in the lack of TonB activity in *exb tol* double mutants. Since TonB-related uptake processes require an energized cytoplasmic membrane (8), the Exb and Tol proteins could alternatively activate TonB such that TonB assumes a conformation which, in turn, induces a conformation of FhuA for the release of receptor-bound colicin M and albomycin into the periplasm and irreversible adsorption of phages T1 and ϕ 80. At high TonB concentrations, a small portion may be in an active conformation to confer T1 sensitivity in the absence of the Exb and Tol proteins. The same mechanism of allosteric regulation via TonB is proposed for the colicin B and D receptor activity.

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LITERATURE CITED

- Bernstein, A., B. Rolfe, and K. Onodera. 1972. Pleiotropic properties and genetic organization of the *tolA,B* locus of *Escherichia coli* K-12. *J. Bacteriol.* **112**:74-83.
- Braun, V., S. Frenz, K. Hantke, and K. Schaller. 1980. Penetration of colicin M into cells of *Escherichia coli*. *J. Bacteriol.* **142**:162-168.
- Davies, J. K., and P. Reeves. 1975. Genetics of resistance to colicins in *Escherichia coli* K-12: cross-resistance among colicins of group B. *J. Bacteriol.* **123**:96-101.
- Davies, J. K., and P. Reeves. 1975. Genetics of resistance to colicins in *Escherichia coli* K-12: cross-resistance among colicins of group A. *J. Bacteriol.* **123**:102-117.
- Eick-Helmerich, K., and V. Braun. 1989. Import of biopolymers into *Escherichia coli*: nucleotide sequences of the *exbB* and *exbD* genes are homologous to those of the *tolQ* and *tolR* genes, respectively. *J. Bacteriol.* **171**:5117-5126.
- Fecker, L., and V. Braun. 1983. Cloning and expression of the *fhu* genes involved in iron(III)-hydroxamate uptake by *Escherichia coli*. *J. Bacteriol.* **156**:1301-1314.
- Fischer, E., K. Günter, and V. Braun. 1989. Involvement of ExbB and TonB in transport across the outer membrane of *Escherichia coli*: phenotypic complementation of *exbB* mutants by overexpressed *tonB* and physical stabilization of TonB by ExbB. *J. Bacteriol.* **171**:5127-5134.
- Hancock, R. E. W., and V. Braun. 1976. Nature of the energy requirement for the irreversible adsorption of bacteriophages T1 and 80 to *Escherichia coli*. *J. Bacteriol.* **125**:409-415.
- Hantke, K., and V. Braun. 1978. Functional interaction of the *tonA/tonB* receptor system in *Escherichia coli*. *J. Bacteriol.* **135**:190-197.
- Hantke, K., and L. Zimmermann. 1981. The importance of the *exbB* gene for vitamin B12 and ferric iron transport. *FEMS Microbiol. Lett.* **12**:31-35.
- Harkness, R. E., and V. Braun. 1989. Colicin M inhibits peptidoglycan biosynthesis by interfering with lipid carrier recycling.

- J. Biol. Chem. **264**:6177–6182.
12. Nagel de Zwaig, R., and S. E. Luria. 1967. Genetics and physiology of colicin-tolerant mutants of *Escherichia coli*. J. Bacteriol. **94**:1112–1123.
 13. Nossal, N. G., and L. A. Heppel. 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J. Biol. Chem. **281**:3055–3062.
 14. Pressler, U., V. Braun, B. Wittmann-Liebold, and R. Benz. 1986. Structural and functional properties of colicin B. J. Biol. Chem. **261**:2654–2659.
 15. Pugsley, A. P., and P. Reeves. 1978. Characterization of group B colicin-resistant mutants of *Escherichia coli* K-12: colicin resistance and the role of enterochelin. J. Bacteriol. **127**:218–228.
 16. Schöffler, H., and V. Braun. 1989. Transport across the outer membrane of *Escherichia coli* via the FhuA receptor is regulated by the TonB protein of the cytoplasmic membrane. Mol. Gen. Genet. **217**:378–383.
 17. Sun, T.-P., and R. E. Webster. 1986. *fii*, a bacterial locus required for filamentous phage infection and its relation to colicin-tolerant *tolA* and *tolB*. J. Bacteriol. **165**:107–115.
 18. Sun, T.-P., and R. E. Webster. 1987. Nucleotide sequence of a gene cluster involved in entry of E colicins and single-stranded DNA of infecting filamentous bacteriophages into *Escherichia coli*. J. Bacteriol. **169**:2667–2674.
 19. Timmis, K. N., and A. J. Hedges. 1972. The killing of sensitive cells by colicin D. Biochim. Biophys. Acta **262**:200–207.