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In Vivo Inhibition of TonB-Dependent Processes by a TonB Box Consensus Pentapeptide

MARGARETA TUCKMAN AND MARCIA S. OSBURNE*

Department of Microbial Genetics and Biochemistry, American Cyanamid Company, Lederle Laboratories, Pearl River, New York 10965

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The TonB box, a conserved pentapeptide sequence found in TonB-dependent colicins and receptors, is thought to interact physically with the TonB protein to facilitate TonB-dependent processes. Strains of *Escherichia coli* were treated in vivo with the synthetic TonB box pentapeptide Glu-Thr-Val-Ile-Val. The pentapeptide inhibited several TonB-dependent processes, including cell growth in low-iron medium, ϕ80 infection, and killing by colicins B and Ia. Two unrelated control pentapeptides had no effect on TonB-dependent processes.

A number of outer membrane receptors, proteins, and colicins have a consensus amino acid sequence, the TonB box, located close to their amino termini (16, 19). These membrane receptors are involved in TonB-dependent processes, such as the uptake of ferrisiderophores and of vitamin B_{12}, and successful infection by phages such as ϕ80 and T1 (for a review, see reference 14). Group B colicins, which have a TonB box, also require the TonB protein for their uptake (1, 15). The existence of the TonB box consensus sequence and genetic evidence that mutations in the TonB box of several receptors or colicins can be suppressed by mutations in the *tonB* gene (4, 8, 12, 17, 18) has led to the hypothesis that the TonB box represents a site of physical interaction of the TonB protein with the various receptor or colicin molecules (8, 18). One way to test this hypothesis was to determine whether an oligopeptide derived from the TonB box could inhibit TonB-dependent processes. Therefore, we treated *Escherichia coli* cells with a synthetic TonB box pentapeptide, Glu-Thr-Val-Ile-Val, derived from the FhuE receptor, which binds ferric coprogen. We then measured several TonB-dependent processes in the presence of this pentapeptide. Two unrelated pentapeptides were used as controls.

The TonB box pentapeptide (116 mg) was purchased from the Protein and Nucleic Acid Chemistry Facility at Yale University. It was stored at room temperature in powder form and dissolved in water as needed at a concentration of 1 mg of pentapeptide per ml. The two control peptapeptides, PP1 and PP2, were Leu-Pro-Pro-Ser-Arg and Val-His-Leu-Thr-Pro, respectively. They were purchased from Sigma Chemical Co., St. Louis, Mo. PP1 and PP2 were handled in the same fashion as the TonB box pentapeptide.

Protection of *E. coli* from the lethal effect of colicins by the TonB box pentapeptide. Colicins B and Ia bind to the iron-regulated outer membrane proteins FepA and Cir, respectively, and require the TonB protein for transport into the cell (1, 15). Because these colicins contain a TonB box (11, 19), we tested the ability of the TonB box pentapeptide to protect *E. coli* from colicin killing. Colicinogenic strains of *E. coli* were obtained from K. Hantke. Colicins were induced and then purified from these strains by a modification (sonication of the producing cells for 2 to 3 min in 15-s pulses) of the method of Hardy and Meynell (7). *E. coli*

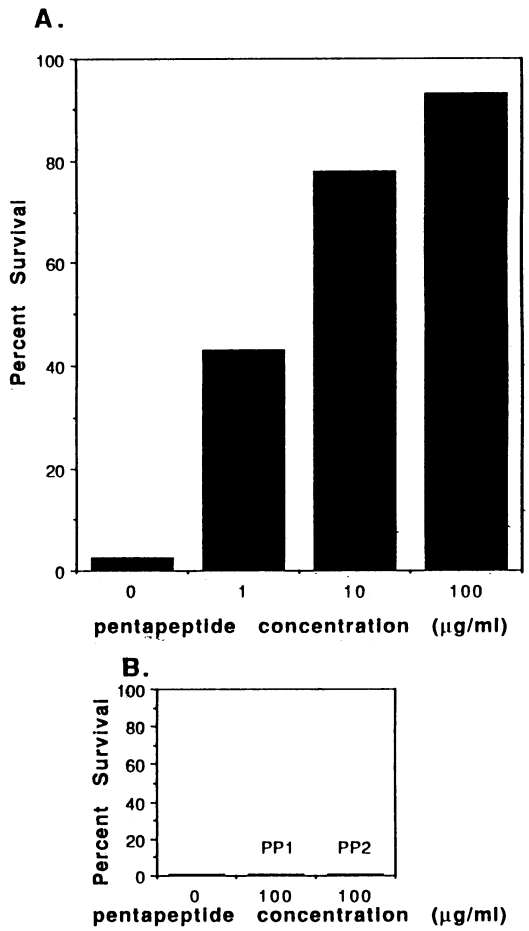

FIG. 1. Protection from colicin killing of *E. coli* by the TonB box pentapeptide. As described in the text, cells were pretreated with the indicated concentrations of pentapeptide and then simultaneously challenged with colicins B and Ia. After overnight growth, the optical density of each culture at 600 nm was measured. Survival is given as the percentage of growth (optical density) of cultures treated with pentapeptide and colicin compared with that of an untreated control culture. Results of treatment with the TonB box pentapeptide (A) and with control pentapeptides PP1 and PP2 (B) are shown.

* Corresponding author.

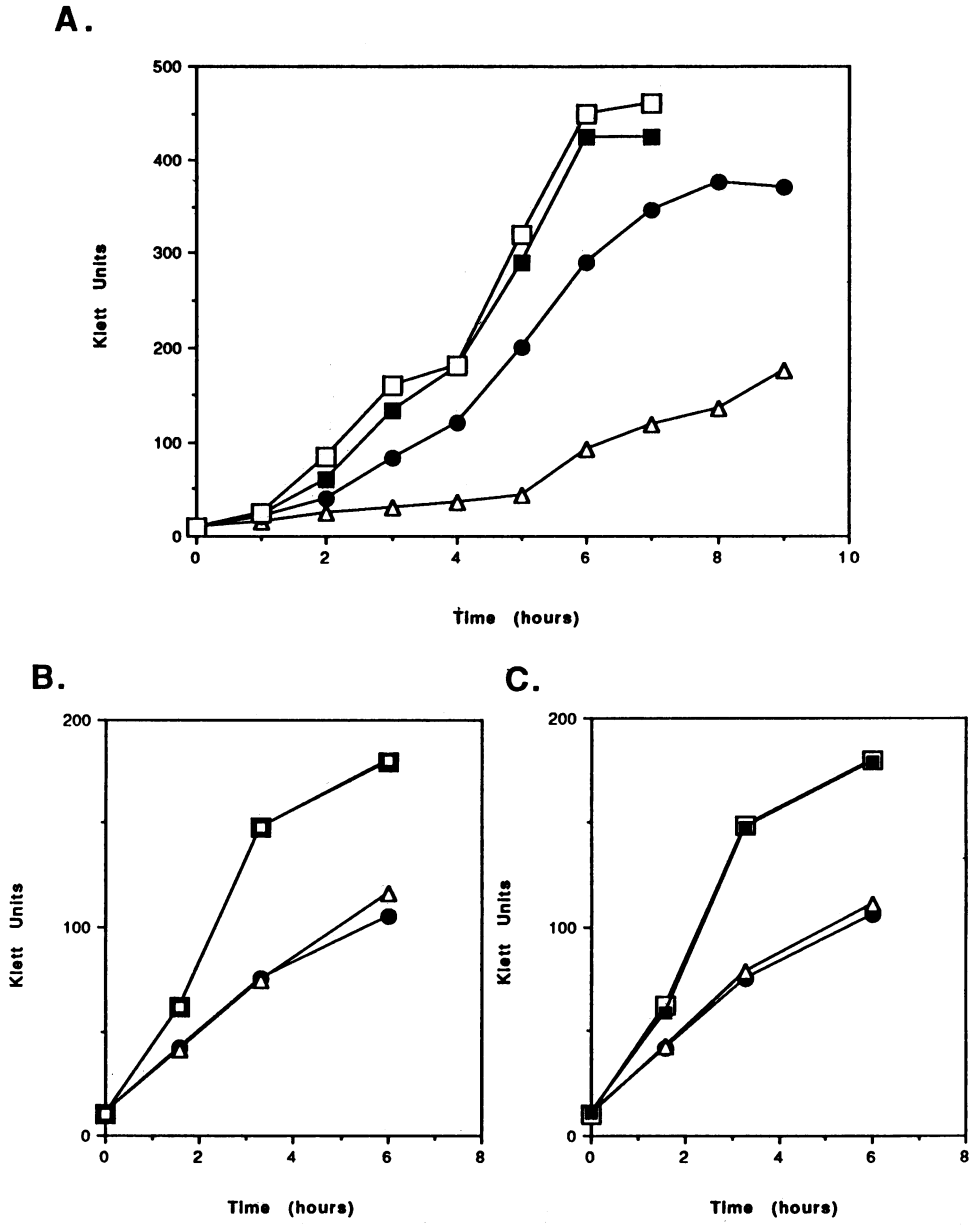

FIG. 2. Inhibition of *E. coli* in low-iron medium in the presence of the TonB box pentapeptide. *E. coli* WW3352 *tonB*$^+$ was grown overnight in LB medium treated with Chelex-100 resin and then diluted in the same medium to a Klett value of 10. Growth at 37°C in the presence of added iron (□), in the presence of added iron and 100 μg of pentapeptide per ml (■), in the absence of added iron or pentapeptide (●), and in the absence of added iron and the presence of 100 μg of pentapeptide per ml (△) was monitored. Results of treatment with the TonB box pentapeptide (A) with control pentapeptide PP1 (B), and with control pentapeptide PP2 (C) are shown.

WW3352 *tonB* was obtained from B. Bachmann. We transduced WW3352 to *tonB*$^+$, using standard P1 transduction techniques (13). The *tonB*$^+$ derivative was inoculated at low density (10^3 to 10^4 cells per ml) in Luria-Bertani (LB) broth (20) at 37°C and treated with various concentrations of the pentapeptide for 1 h at 37°C. Cells were then challenged with a mixture of colicins B and Ia and incubated overnight at 37°C. Cell growth was determined by measuring the A_{600}. Figure 1A shows that 100 μg of the TonB box pentapeptide per ml protected cells from the lethal effect of the colicins. By contrast, the control pentapeptides PP1 and PP2 had no protective effect (Fig. 1B). The experiment was repeated, with similar results, with four unrelated strains of *E. coli* K-12 (data not shown).

Protection of *E. coli* from phage ϕ80 infection by the TonB box pentapeptide. Successful ϕ80 infection of *E. coli* requires binding of the phage to the FhuA receptor, a siderophore receptor used for the binding and transport of ferrichrome (5, 10). Infection also requires an active TonB protein. To assess the effect of the pentapeptide on ϕ80 infection, we used *E. coli* H455, an *aro* mutant strain (obtained from Nigel Curtis) which we converted to *aro*$^+$ by P1 transduction. Cells were grown at 37°C in LB broth containing 10 mM Mg^{2+}. An equal volume of pentapeptide solution (100 μg/ml)

TABLE 1. Inhibition of φ80 infection of *E. coli* by the TonB box pentapeptide[a]

Expt no. and PP	MOI[b]	Cell survivors without PP		Cell survivors with PP	
		CFU/ml	%[c]	CFU/ml	%
1. TonB box	5	2.8×10^7	7.0	7.9×10^8	197.2
2. TonB box	50	2.8×10^7	4.1	7.9×10^8	116.0
3. TonB box	100	1.9×10^7	1.6	1.4×10^8	11.7
4. Controls					
PP1	5	1.4×10^8	7.7	1.5×10^8	8.3
PP2	5	1.4×10^8	7.7	1.2×10^8	6.6

[a] Strain H455 *aro*$^+$ was infected with φ80 as described in the text. PP, pentapeptide.
[b] MOI, multiplicity of infection.
[c] The percentage of survivors is based on the number of cells present in an uninfected control culture not treated with pentapeptide. Titers of the control (uninfected) cultures were determined at the time infected cultures were titered for cell survival. For experiments 1, 2, and 3, the control titers were 4×10^8, 6.8×10^8, and 1.2×10^9 CFU/ml, respectively. The control titer for experiment 4 was 1.8×10^9/ml.

was then added, and the culture was incubated for an additional hour at 37°C. The cells were then infected with φ80 (2×10^8 cells per ml; multiplicity of infection as indicated) and incubated at 37°C for an additional 30 min. At this point, the cells were washed twice in 0.85% saline, resuspended in 0.85% saline, and plated for survivors. Pretreatment with the pentapeptide resulted in a significant increase in the number of survivors (Table 1), suggesting that interference with activity of the TonB protein by the pentapeptide blocked successful phage infection. Again, the control pentapeptides PP1 and PP2 had no effect on successful phage infection (Table 1).

As an additional control, we tested the effect of the TonB box pentapeptide on infection by phage T5. This phage requires the FhuA receptor for binding to the cell surface but does not require the TonB protein for successful infection (6). By using the protocol described above for φ80 infection, cells were pretreated with the TonB box pentapeptide and then infected with phage T5. As expected, the TonB box pentapeptide had no significant effect on T5 infection; cell survival was 2.6% in the absence of pentapeptide and 5.1% in the presence of pentapeptide. These experiments were performed at a multiplicity of infection of 5 phage per cell.

Growth inhibition of *E. coli* by the TonB box pentapeptide in low-iron medium. The TonB protein is required to facilitate the transport of ferrisiderophores from outer membrane receptors into the cell (for a review, see reference 14). If the TonB protein was inactivated by the TonB box pentapeptide, cell growth would be inhibited at Fe^{3+} concentrations of less than 10^{-6} M. To test this hypothesis, *E. coli* WW3352 *tonB*$^+$ was first grown overnight at 37°C in LB medium pretreated with Chelex-100 resin (3). This low-iron medium cannot support the growth of *E. coli tonB* mutants. Cells were diluted to a density of approximately 2×10^7 cells per ml in low-iron LB medium, and growth at 37°C was monitored with a Klett-Summerson colorimeter (green filter) in the presence and absence of 10^{-6} M $FeCl_3$ and 100 μM pentapeptide. Figure 2A shows that addition of the TonB box pentapeptide to the medium had no deleterious effect when ample iron was present. In low-iron medium, when the TonB-dependent iron uptake system was in effect and no pentapeptide was added, growth was somewhat slower. When the pentapeptide was added to the low-iron medium, however, growth was inhibited for at least 5 h. After 5 h the growth rate slowly increased, suggesting that the pentapeptide was metabolized, possibly by means of uptake through the oligopeptide permease (*opp*) system (9) and/or by protease degradation. Figure 2B and C shows that the control pentapeptides PP1 and PP2 had no inhibitory effect on cells growing in low-iron medium.

The experiments described above provide supportive evidence that the TonB protein of *E. coli* interacts directly with the TonB box present on outer membrane receptors and colicins which are known to require TonB for transport functions. Three processes, infection by colicins B and Ia, infection by phage φ80, and cell growth in low-iron medium, were all specifically inhibited by a synthetic pentapeptide containing amino acids derived from the TonB box consensus sequence of the FhuE receptor. The TonB protein is required for all of these functions. Inhibition by the pentapeptide strongly suggests that the pentapeptide binds directly to the TonB protein and prevents its direct interaction with receptors and colicins. These findings are consistent with recent direct evidence of specific interaction between the TonB protein and the FhuA outer membrane receptor for ferrichrome-iron (2).

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