# AN ASSAY TO DETECT INHIBITORS OF BACTERIAL IRON TRANSPORT

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Although iron is an abundant element, it is not readily available to microorganisms because it is poorly soluble at physiological pH[1]. Furthermore, in serum and secretory fluids, iron is tightly sequestered by host carrier proteins. For these reasons, the available supply of iron to infecting microorganisms is limited, a factor which can prevent microbial growth[1,2]. In fact, a positive correlation has been made between successful infection by microorganisms which grow extracellularly, and their ability to acquire iron from host proteins. Thus, iron acquisition by pathogenic bacteria has been recognized as a virulence factor[2~4].

The concentration of free iron in human serum ($10^{-18}$ M)[5] is too low for iron to enter bacteria by passive diffusion, for which a concentration of at least $10^{-6}$ M is required[4]. To circumvent this problem, many bacteria elaborate and transport siderophores, small molecules that chelate $Fe^{+3}$. The production of siderophores appears to enhance the virulence of bacteremic strains (for a review, see reference 2). Therefore, the iron uptake system of bacteremic strains may furnish an effective target for novel antibacterial agents. Here we present a screening system designed to detect inhibitors of bacterial iron transport.

## Rationale for the Design of an Assay to Detect Inhibitors of the TonB Protein

Enteric organisms synthesize a variety of siderophores which are utilized for iron transport. Furthermore, they can make use of many siderophores produced by other microorganisms, such as fungi[6]. It is therefore not practical to target a particular siderophore or siderophore receptor for antimicrobial chemotherapy. On the other hand, the TonB protein is required for the translocation of all usable ferrisiderophores back into the cell[7]. Thus the TonB protein is absolutely required for growth of *E. coli* in low-iron media, regardless of the number and type of siderophores available[8]. For this reason, it seemed reasonable to develop an assay to detect inhibitors of the TonB protein. Such inhibitors are potential antibacterial agents.

## The TonB Assay

An obvious assay strategy for the detection of TonB inhibitors would be to look at the ability of a compound to inhibit the growth of *E. coli* in low iron medium. Although conceptually straightforward, this assay is cumbersome as a high throughput assay, since low-iron medium is variable, and must be carefully quality controlled. Therefore we found it advantageous to prescreen compounds in a two-part assay, to reduce the number of candidate compounds to be tested in low-iron medium.

In the first assay, the Rescue Assay, compounds which rescued *E. coli* from the lethal effects of TonB-dependent colicins B and Ia are identified. In addition to its role in iron transport, the TonB protein is required for other cellular functions, including transport of vitamin $B_{12}$ and certain colicins from specific outer membrane receptors to the inside of the cell, and irreversible adsorption of phages such as T1 and $\phi$ 80 to the cell surface (reviewed in reference 8). *ton*B mutants of *E. coli* are deficient in these and other processes, and are resistant to colicins B and Ia. If TonB function were inactivated, *E. coli* would be immune to TonB-dependent colicins. In the assay, two different colicins with different outer membrane receptors were used, so that any compound which rescued the cell would probably be directed against the TonB protein rather than against a specific colicin receptor. Note that this assay has the advantage that it is performed in standard LB medium, and does not require iron-poor medium. The assay is carried out as follows. Test compounds or culture broths are spotted (in 10 $\mu$l volumes) on LB agar plates[9], pH 7.0, and incubated at room temperature for 30 minutes to allow diffusion of the compounds into the agar. Compounds or culture broths form a concentration gradient as they diffuse out from the central spot. Agar plates are then overlayed, using LB soft agar, with *E. coli* H455 (from N. CURTIS, (*aroB malT tsx thi* $\Delta$(*prolac*), converted to *aro*+ by

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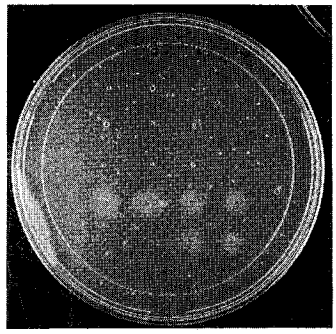
Fig. 1. The colicin rescue assay for the detection of TonB inhibitors.



The assay was performed as described in the text. The zones of bacterial growth on the assay plate represent rescue of *E. coli* from the lethal effects of colicins B and Ia, following treatment with culture broths containing unknown components.

standard P1 transduction techniques[10]), which is grown at 37°C with shaking from a 1:20 dilution of an overnight culture, to a Klett of 50 (Klett-Summerson colorimeter, green filter). Approximately $2.5 \times 10^7$ cells are used per ml of soft agar. Plates are incubated further for 1 hour at 37°C to allow time for potential inactivation of the TonB protein, and then overlayed with colicins Ia and B in LB soft agar. Colicinogenic strains were obtained from K. HANTKE, and colicins were induced and purified from these strains as described previously[11]. Colicins were pretitered so that the smallest amount of colicin resulting in complete killing of a lawn was used. After overnight incubation at 37°C, and then again after an additional 24 hours incubation, plates are inspected for zones of growth rescue. An example of an assay plate is shown in Fig. 1.

Compounds positive in the rescue assay are put through a secondary assay, the Zone of Inhibition Assay, which tests the ability of a compound to inhibit growth of *E. coli* strain H455, specifically on low-iron agar plates. Positive compounds should produce a zone of inhibition on the dipyridyl-containing plate, but not on the LB plate, consistent with inhibition of the TonB protein, which is required for growth in low-iron medium, but not required for growth in iron-replete medium. We defined low-iron medium as LB agar containing 200 $\mu$M 2,2'-dipyridyl, on which *ton*B mutants of *E. coli* were unable to grow. *E. coli* cells are grown overnight in low-iron medium, diluted, and regrown in the same medium to a Klett of 50. A lawn of cells is applied to the low-iron plate and to a standard LB (iron-replete) control plate. Compounds of interest are spotted on the lawns, plates are incubated overnight at 37°C, and then inspected for zones of inhibition.

Table 1. Effect of the TonB box pentapeptide in the rescue and zone of inhibition assays.

| Amount of pentapeptide[a] | Rescue assay[b] | Zone of inhibition assay[b] |
|---|---|---|
| 100 | 17[c] | 25[c] |
| 10 | 11 | 10 |
| 1 | 8 | 0 |
| 0.1 | 0 | 0 |

[a] The amount of pentapeptide applied to the assay plate is expressed in nanograms (ng).
[b] The assays were performed as described in the text. The "low-iron" plate in the zone of inhibition assay contained 200 $\mu$M 2,2'-dipyridyl, resulting in a free iron concentration of $< 10^{-6}$ M.
[c] Numbers represent the size, in millimeters, of the rescue or growth inhibition zones.

Behavior of a Variety of Drugs in the TonB Assay

A variety of antimicrobial agents have been tested in the TonB assay (data not shown). We found that, for unknown reasons, some antibiotics were slightly positive in the rescue assay at sub-inhibitory concentrations. At these concentrations, however, the compounds were negative in the zone of inhibition assay. This result reinforces the necessity of both parts of the assay.

It is known that iron receptor proteins and colicins which require TonB for transport share a consensus 5 amino acid sequence, the TonB box, which is thought to interact with the amino terminal portion of the TonB protein to facilitate TonB-dependent processes[12,13]. We tested the synthetic TonB box pentapeptide glu-thr-val-ile-val, (purchased from the Protein and Nucleic Acid Chemistry Facility at Yale University), and found that the pentapeptide was active in the TonB assay (Table 1). This result was expected, since previous experiments[7] indicated that this pentapeptide interfered with several TonB-dependent activities *in vivo*.

## Discussion

We have developed an assay to detect inhibitors of the *E. coli* TonB protein. A TonB box consensus amino acid pentapeptide served as a positive control for the assay. Using this assay, it may be possible to identify inhibitors of iron

transport for pathogenic bacteria which require a functional TonB protein.

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