

# Epsilon Toxin from *C. perfringens*: Characterization of Strain Genotype, Phenotype and Toxin Sensitive Cell Line

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## Abstract

**Introduction:** Epsilon toxin is one of the most potent clostridial toxins known. It is a USDA and HHS select agent toxin and is included on the NIAID list of Category B Priority Pathogens. Not all *C. perfringens* strains contain the epsilon gene or express the protein, thus it is critical to know the genotype and phenotype of strains in any collection in order to segregate the epsilon positive strains from the remaining strains. One hurdle in the field is identification of cell lines suitable for characterization of the biological activity of this protein. We sought to address these needs by a) characterizing a collection of strains for the presence of the gene and expression of epsilon protein and b) identification of cell line(s) that were sensitive to the protein.

**Methods:** The genotype of *C. perfringens* strains was determined using a multiplex PCR method to detect 7 toxin genes. Culture supernatants from these strains were tested for expression of epsilon protein and other toxins known to be produced by *C. perfringens*. Cell lines were tested for sensitivity to epsilon toxin using pore forming and biochemical assays.

**Results:** 35 strains were tested for their genotype. Of these, 54% of the collection was Type A, 2.9% of the collection was Type B, 20% of the collection was Type D, and 11.4% of the collection was Type E. 8 strains were epsilon positive by genotyping yet only 4 of these strains made the protein at detectable levels. Cell lines from the ATCC collection were tested for sensitivity to epsilon toxin and at least one cell line was shown to be sensitive.

**Conclusions:** The genotype of the *C. perfringens* collection at ATCC® was determined and the strains were typed according to the combination of toxin genes present. Strains were identified that both possess the epsilon gene and express the protein. A cell line was identified that was sensitive to the protein and can be used to characterize the biological activity of the protein.

## Introduction

*Clostridium perfringens* are gram positive, obligate anaerobes that produce multiple toxins and form endospores. These toxins can cause human disease such as food poisoning, gas gangrene and enterotoxemia. To date >15 different toxins have been identified in the genome of *C. perfringens*. Depending on the combination of these toxins, *C. perfringens* is separated into one of five types (A-E). Type A and C strains are the primary disease-causing strains in humans while Types B, D, and E strains are responsible for necrotic enteritis or enterotoxemia in animals. Type A strains express the  $\alpha$  toxin which is encoded by the *cpa* gene and are primarily responsible for gangrene and food poisoning in humans. Type B strains express  $\alpha$ ,  $\beta$  and  $\epsilon$  toxins encoded by the *cpa*, *cpb*, and *etx* genes. Type C strains express  $\alpha$  and  $\beta$  toxins, Type D strains express  $\alpha$  and  $\epsilon$  toxins, and Type E strains express  $\alpha$  and  $\iota$  (iota) toxins. Epsilon toxin is a USDA and HHS select agent toxin and is included on the NIAID list of Category B priority pathogens making it an important protein to characterize.

The primary goals of this project are to a) genotype the *C. perfringens* strains included in the ATCC collection for the presence or absence of toxin genes, b) determine whether these toxins are expressed at detectable levels, and c) identify cell lines that may be sensitive to epsilon toxin. The data collected will provide outside researchers the ability to select the best strain for their research program and guide us in our acquisition strategy for obtaining new isolates of *C. perfringens*.

## Materials and Methods

### Bacterial strains and culture conditions:

Bacterial strains were obtained from the ATCC Bacteriology collection and were grown in reinforced clostridial media ATCC #1053. All strains were cultured under anaerobic conditions at 37°C. Cultures were plated on Tryptic Soy Agar with 5% defibrinated sheep blood (Becton Dickinson 236950), and single colonies were picked for expansion and isolation of genomic DNA. The clarified culture supernatants were stored at -80°C for use in western blots.

### Multiplex PCR genotyping of *C. perfringens*:

Each strain was tested for the presence or absence of the toxin genes *cpa*, *cpb*, *cpb2*, *cpe*, *iap*, and *etx* using a multiplex PCR procedure as previously reported with some modifications (Garmory 2000, Meer 1997). We added *pfoA* to our multiplex using primers described in Fisher et al, 2006. Strains known to possess each of the toxin genes were used as positive controls. A DNA standard ladder containing the PCR products of each toxin gene was generated by combining the resulting PCR products from individual reactions using the multiplex primers. Briefly, each toxin target was amplified by PCR in singleplex mode using the primers from the multiplex reaction. The PCR products were purified individually and then combined to generate the DNA standard ladder. In some cases, it was difficult to determine whether the *cpb* gene was present. To confirm the presence or absence of the *cpb* gene, the entire collection was screened again using a new set of primers designed to amplify the *cpb* gene. The PCR product of ~1200 base pairs was observed in the positive control and strains containing the *cpb* gene.

### Measurement of *C. perfringens* toxin expression:

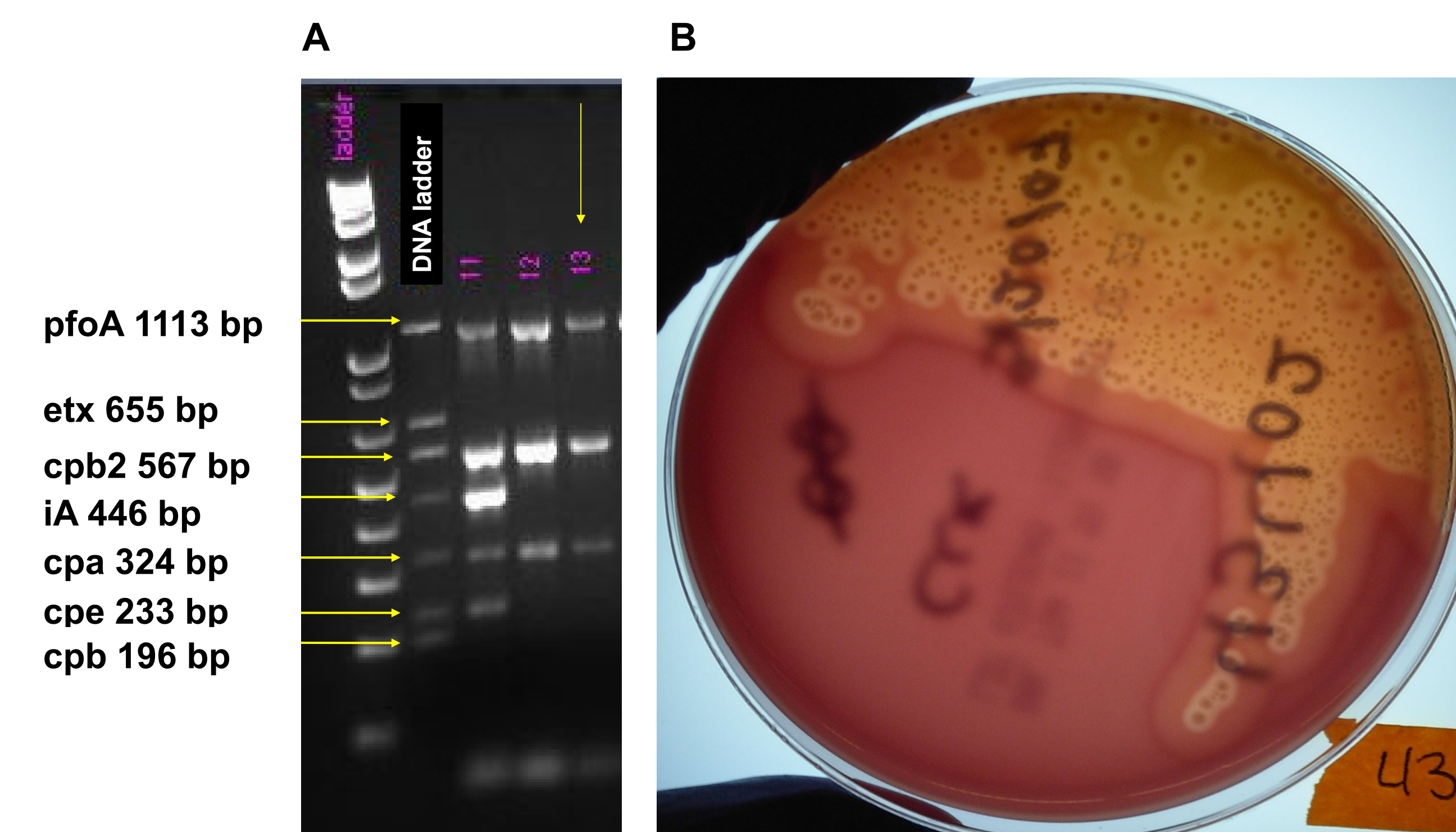
Culture supernatants from the overnight growths were mixed with an equal amount of sample reducing buffer containing  $\beta$ -mercaptoethanol and were run on Invitrogen 4-20% SDS-PAGE gels under denaturing conditions. The proteins were transferred to PVDF membranes and probed with polyclonal antibodies to Cpb (AbD Serotec) or Etx (BEI Resources NR-865) proteins. The bands were visualized using anti-rabbit antibodies conjugated with IRDye 680 and imaged on the Li-Cor Odyssey (Li-Cor Biosciences). The positive control Cpb toxoid and Etx toxoid proteins were obtained from the National Institute of Biological Standards and Control, United Kingdom.

### Identification of epsilon sensitive cell lines:

Cytotoxicity of epsilon toxin was determined using the method of Schmitt 1991. MDCK (NBL-2) (ATCC® CCL-34™) cells were cultured in Eagle MEM and 10% heat inactivated fetal bovine serum. Briefly, cells were plated in 96-well plates and cultured overnight. The following day, the culture media was removed and various concentrations of activated epsilon toxin (BEI Resources NR-4670) or epsilon protoxin (BEI Resources NR-856) was diluted in media, added to the cells and cultured at 37°C for 48 hours. The media containing toxin was removed and the cells were fixed with 10% formalin. The fixed cells were stained with 0.13% crystal violet for 2 hours. Residual stain was removed and excess dye was washed away with water. The dye was solubilized with 70% methanol and the absorbance at 600 nm was read on a SpectraMax plate reader.

## Results

### Multiplex PCR Genotyping of the *C. perfringens* Collection



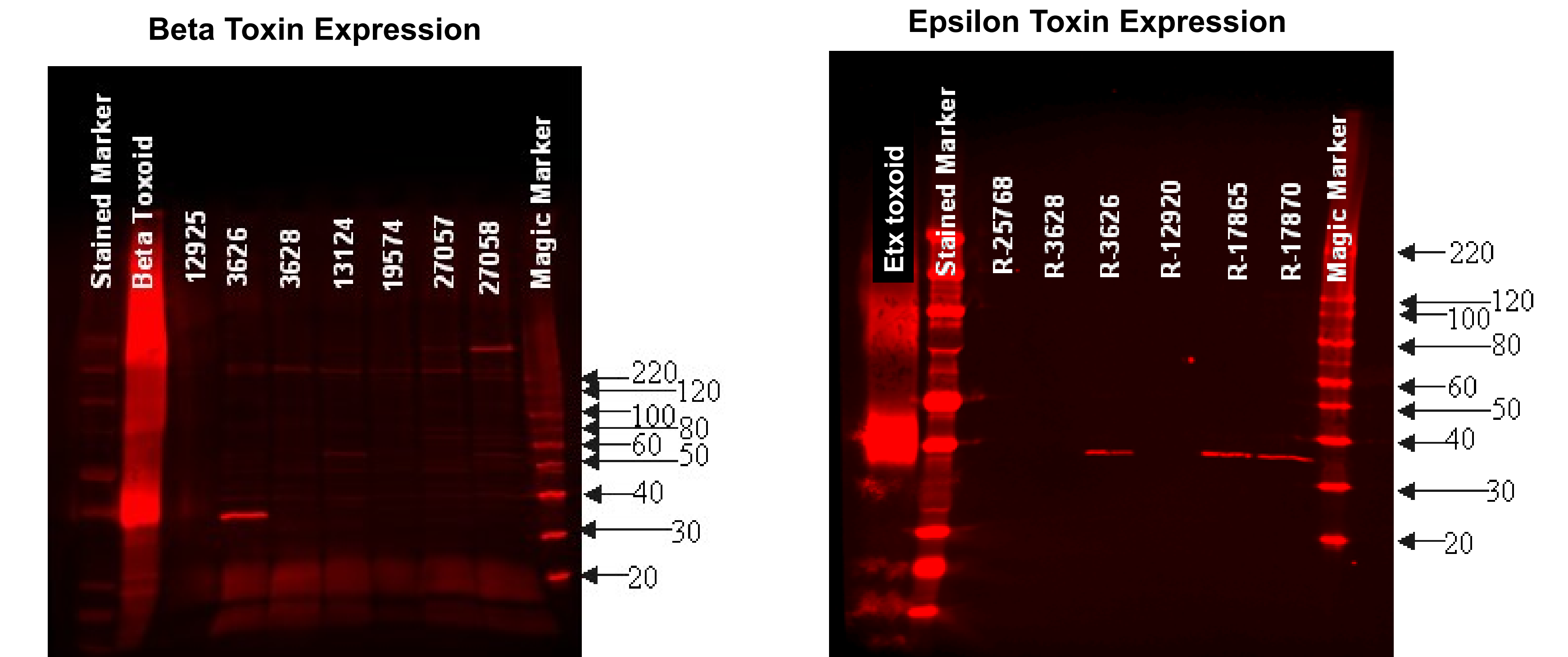
**Figure 1. Genotype and Phenotype of *C. perfringens* strains.** A) Each strain in the collection was tested for the presence or absence of toxin genes using a multiplex PCR reaction. B) The genotype and phenotype of a strain are correlated. The strain marked with the yellow arrow was genotyped and shown to have *pfoA*, *cpb2*, and *cpa* genes. When the same strain was plated on TSA plates containing sheep blood, the activity of Cpa and PfoA can be seen by the zone of hemolysis surrounding each colony. Perfringolysin (PfoA) causes a more complete zone of hemolysis, while  $\alpha$  toxin (Cpa) causes the hazy or incomplete zone of hemolysis.

ATCC® Strain	<i>cpa</i>	<i>cpb</i>	<i>etx</i>	<i>iA</i>	<i>cpe</i>	<i>cpb2</i>	<i>pfoA</i>
3626™	+	+	+	-	-	+	+
3628™	+	-	+	-	-	-	+
3629™	+	-	-	-	-	-	+
3631™	+	-	+	-	-	-	+
8009™	+	-	-	+	-	-	+
9856™	+	-	-	-	-	+	+
10543™	+	-	-	-	-/+	+	+
12915™	+	-	-	-	+	-	-
12916™	+	-	-	-	+	-	-
12917™	+	-	+	-	+	-	-
12918™	+	-	-	-	+	-	-
12919™	+	-	+	-	+	-	-
12920™	+	-	-	-	+	-	-
12921™	+	-	-	-	-/+	-	-
12922™	+	-	-	-	+	-	-
12924™	+	-	-	-	+	-	-
12925™	+	-	-	-	+	-	-
13124™	+	-	-	-	-	-	+
14809™	+	-	+	-	+	-	-
17865™	+	-	+	-	-	-	+
17870™	+	-	+	-	-	+	+
19574™	+	-	-	-	+	+	+
25768™	+	-	-	-	-/+	-	-
27057™	+	-	-	-	-	-	+
27058™	+	-	-	-	-	-	+
27059™	+	-	-	-	-	+	+
27324™	+	-	-	+	+	+	+
29348™	+	-	-	-	-	+	+
43402™	+	-	-	-	-	+	+
43403™	+	-	-	-	-	-	+
VPI 1589	+	-	-	+	+	+	+
VPI 10339	+	-	-	+	+	+	+
19406™ <i>C. TETANI</i>	-	-	-	-	-	-	-
17874™	+	-	-	-	+	+	-
21510™	+	-	-	-	+	-	-
10388™	+	-	-	-	-	-	+

**Table 1. Genotype of each strain in the ATCC collection.** Each strain that was available at the time of testing was typed using the multiplex PCR. *Clostridium tetani* was used as a negative control and two strains from the VPI collection were used as type E positive controls.

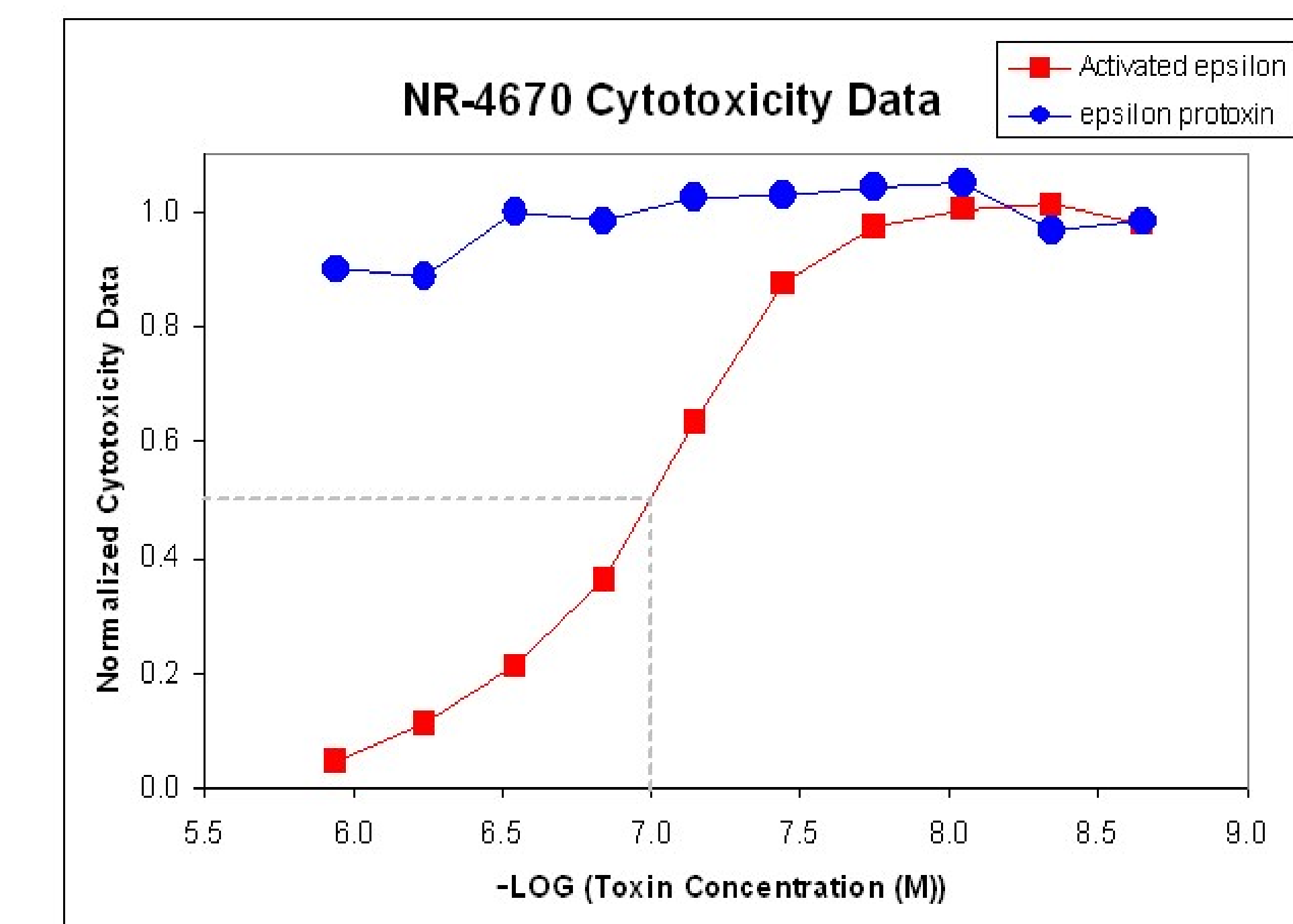
TYPE	% OF COLLECTION
A	54.3%
B	2.9%
C	0.0%
D	20.0%
E	11.4%
pfoA +	60.0%
Untyped	11.4%

### Phenotype of the *C. perfringens* Collection.



**Figure 2. Expression of Beta (cpb) and Epsilon (etx) toxins by *C. perfringens* strains.** The culture supernatants from strains grown in reinforced clostridial media were analyzed by western blot for the expression of Cpb and Etx proteins. ATCC® Strain 3626™ was the only one shown to express Cpb toxin while strains 3626™, 17865™, 17870™, and 3631™ expressed Etx toxin.

### Cytotoxicity of the Protoxin and Activated Forms of Epsilon Toxin



**Figure 3. Cytotoxicity of epsilon toxin on MDCK cells.** Serial dilutions of purified epsilon protoxin (NR-856) and activated toxin (NR-4670) were incubated with MDCK cells for 48 hours. Cells that survived intoxication were fixed with formalin, stained with crystal violet, and the absorbance read on a plate reader. Values near 1 indicate cell survival while values near 0 indicate cell death. These data show that the protoxin is not toxic to these cells while the trypsin activated protein is toxic.

## Conclusions

- The ATCC *C. perfringens* collection contains representatives from 4 of the 5 toxin types described as determined by genotypic and phenotypic methods. Type C is under represented in the collection.
- Multiple strains were shown to possess the Epsilon toxin (*etx*) gene and some of these strains express the protein.
- MDCK cells were sensitive to the trypsin activated Epsilon toxin, but not the protoxin.

## References

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