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Development of a Vaccine Against Clostridium difficile Infection (CDI): Design, Purification, and Biological Activities of the Recombinant Toxin 1 Antigens

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Development of a Vaccine Against *Clostridium difficile* Infection (CDI): Design, Purification, and Biological Activities of the Recombinant Toxin Antigens

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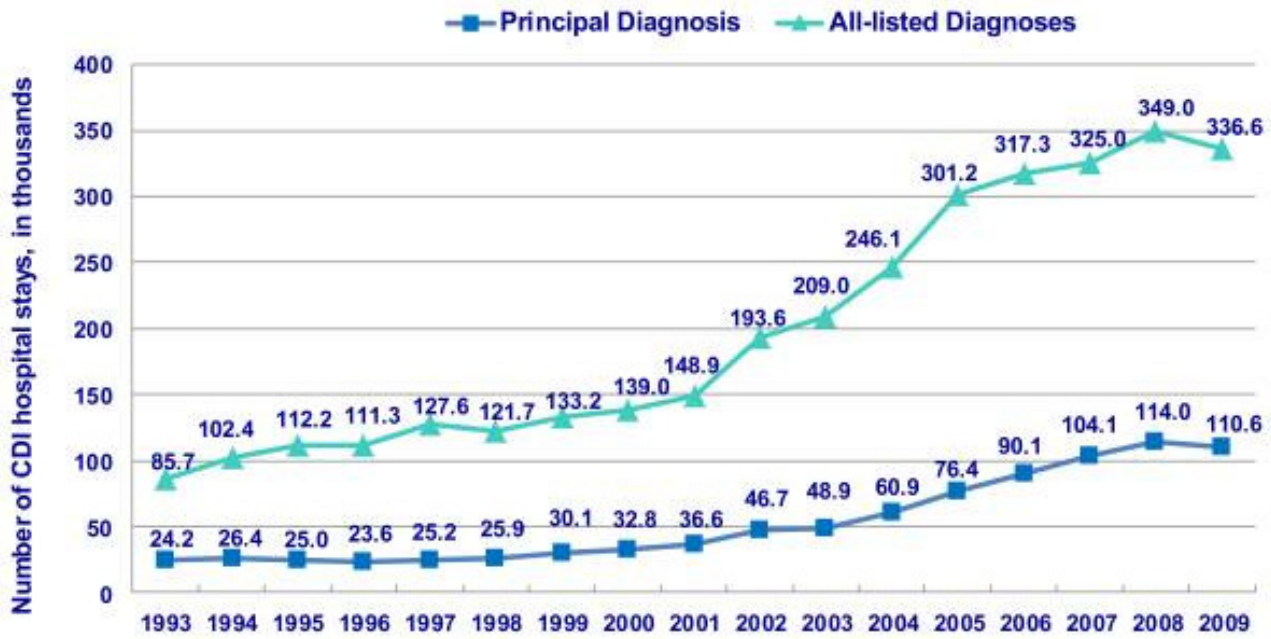
Clostridium difficile Infection (CDI)

- *Clostridium difficile* infection (CDI) is a leading cause of nosocomial diarrhea
- CDI can lead to colitis, toxic megacolon, systemic inflammatory response syndrome, and death
- 500,000 cases/year in the US



J Med Microbiol. 2011 Aug;60(Pt 8):1070-9.
Nat Rev Microbiol. 2009 Jul;7(7):526-36.
Healthcare Cost and Utilization Project (HCUP) Statistical Briefs Statistical Brief #124, May 2012.

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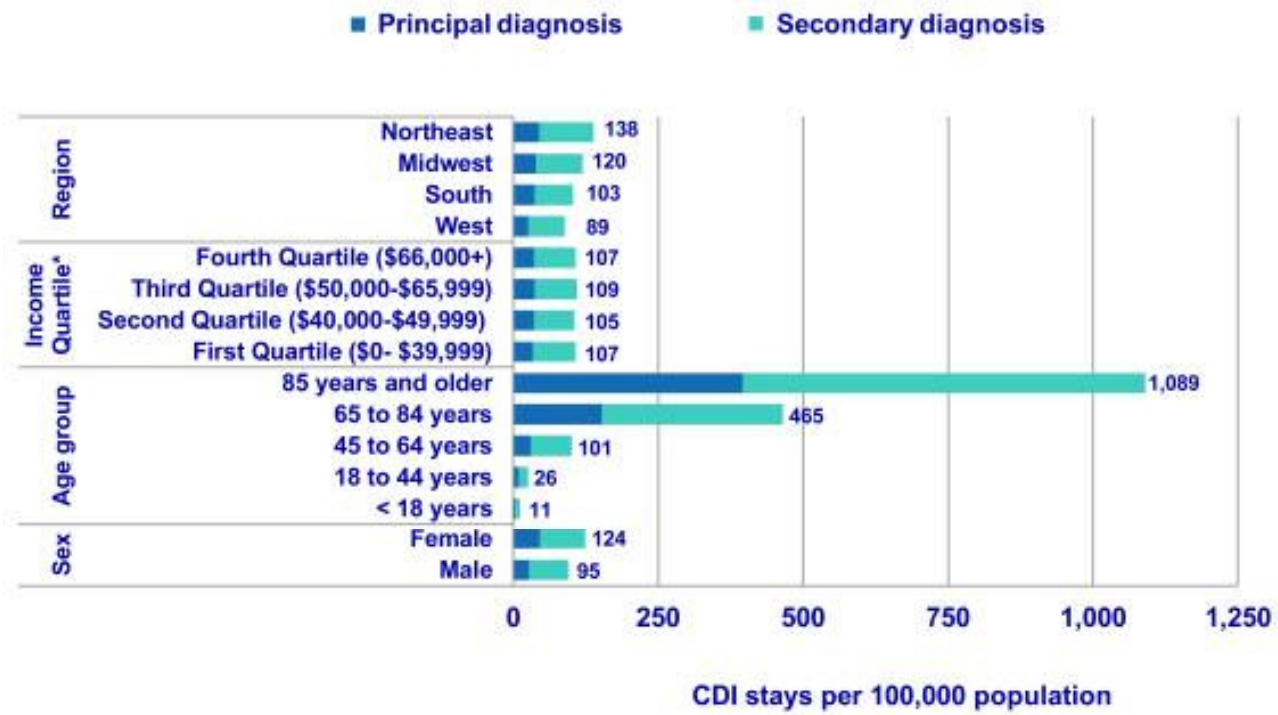
Clostridium Difficile Infections (CDI) 2009



- Substantial morbidity among patients:
  - Elderly (age >65 years)
  - Immunocompromised
  - Undergoing prolonged hospitalization
  - Receiving broad-spectrum antibiotics and/or proton pump inhibitors

Healthcare Cost and Utilization Project (HCUP) Statistical Briefs Statistical Brief #124, May 2012.

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C. difficile Virulence Factors

- *Clostridium difficile* is an anaerobic spore-forming gram-positive bacterium

- The main virulence factors are two clostridial toxins: TcdA and TcdB

- Asymptomatic carriers of *C. difficile* have significantly higher serum anti-toxin IgG levels as compared with patients who develop primary or recurrent CDI (J Med Microbiol. 2011 Aug;60(Pt 8):1070-9)

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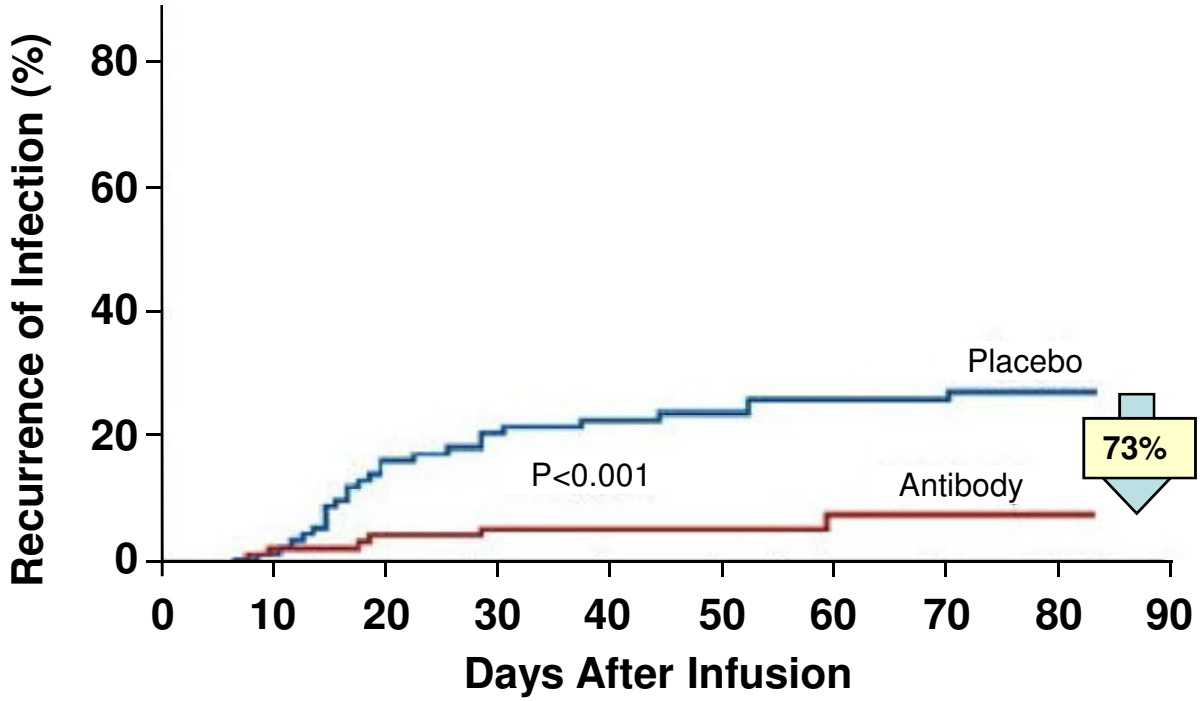
Monoclonal Antibodies against TcdA and TcdB Reduce Recurrence of CDI in PII Clinical Study



- Treatment with monoclonal antibodies CDA1 and CDB1 reduces the rate of disease recurrence following SoC treatment (metronidazole or vancomycin)

N Engl J Med. 2010 Jan 21;362(3):197-205.

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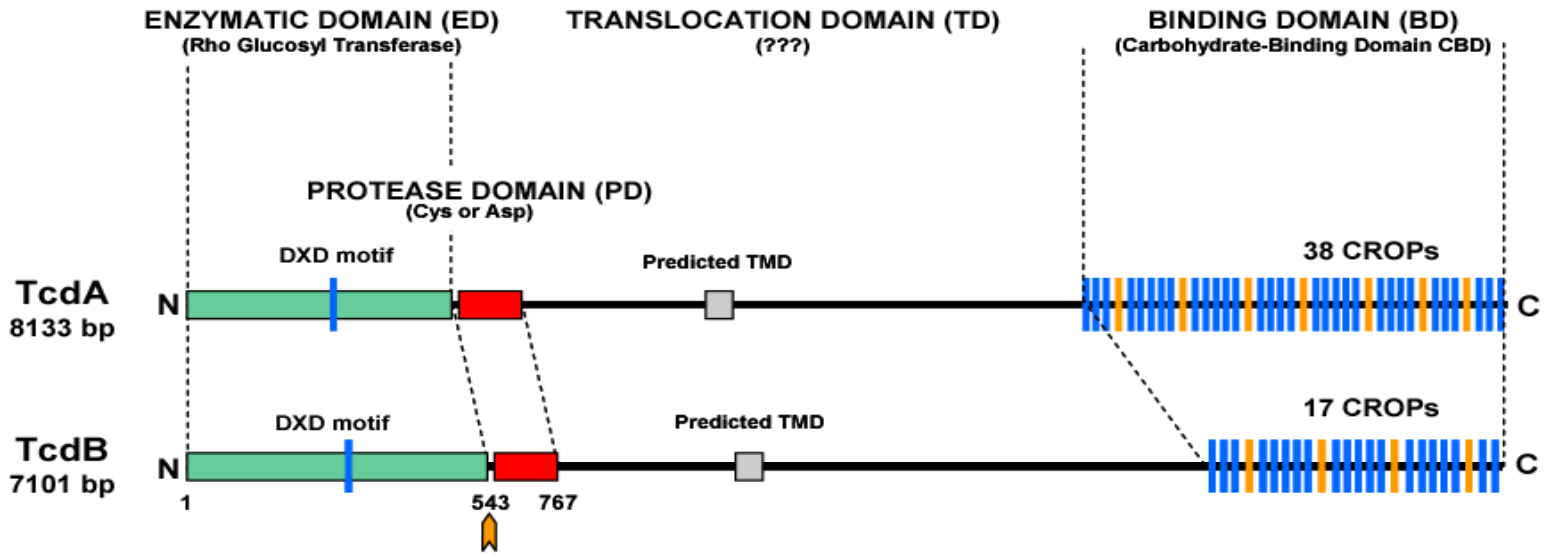
Domain Structure of *C. difficile* TcdA and TcdB



- TcdA and TcdB are large (~**300 kDa**) proteins composed of N-terminal enzymatic domain (ED), glucosyl transferase, the middle region translocation domain (TD) and C-terminal, highly repetitive carbohydrate binding domain (CROPS).
- Native, formalin inactivated Toxoid A/B vaccine is safe and immunogenic, currently in Phase II (Sanofi-Aventis)
- Recombinant CROPS region, chimeric antigens, and DNA vaccines induced neutralizing antibodies are under evaluation as vaccine candidates

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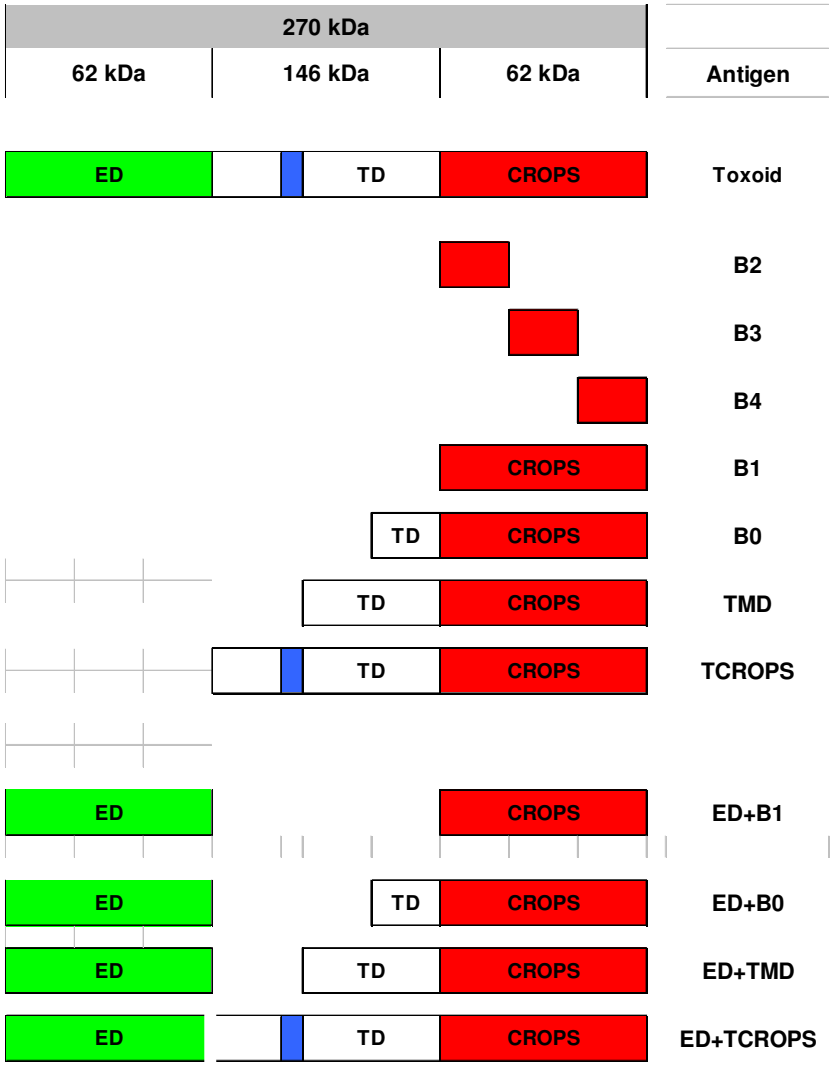
Design of TcdB Recombinant Fragments Used in the Study



Objectives of fragmentation study:

1. Which CROPS segments induce protective antibodies?
2. Does TD play a role in a development of protective immunity?
3. Does ED contain additional neutralizing epitopes?

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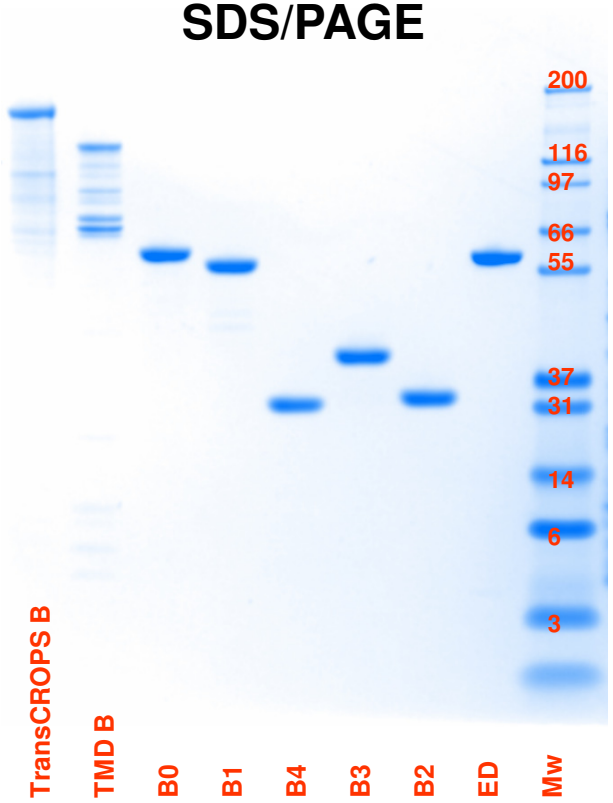
Expression and Purification of Recombinant Fragments of TcdB

SDS/PAGE



Purification yields

Fragment name	Molecular weight kDa	Yield* mg/liter
TransCROPS B	207	39
TMD B	142	50
B0	68	822
B1	62.4	582
B2	31.6	1836
B3	39.1	686
B4	32	800
ED B	63.9	505

* Assuming 20% biomass, after two-step purificaiton

- All antigens were purified in the two-step process using Ni-affinity column followed by anion exchange chromatography
- Highly purified antigens were obtained (SDS/PAGE) with purification yields 0.5-2 g/L for shorter fragments and 40-50 mg/L for longer antigens

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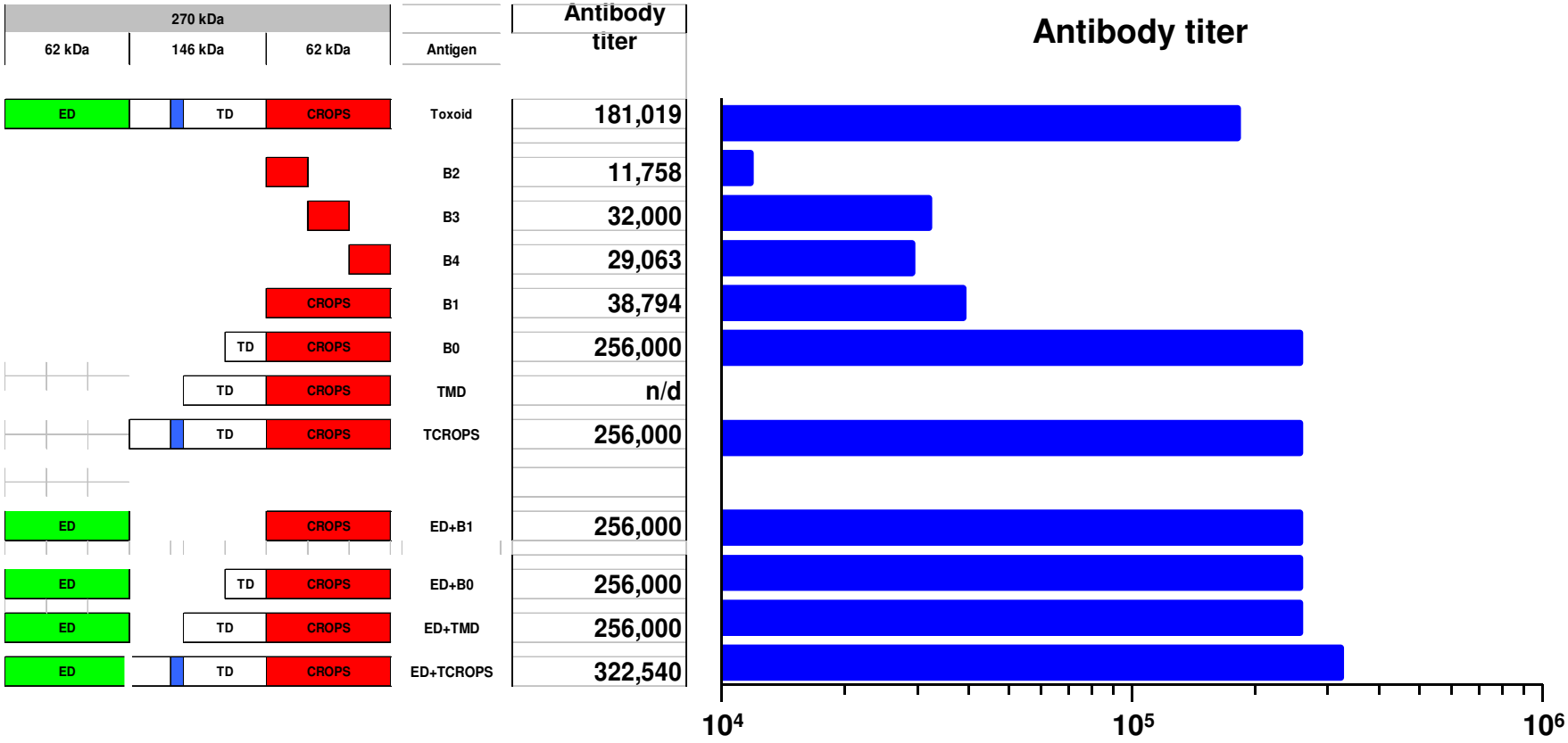
Antibody Titers in Hamsters Immunized With TcdB Antigens



- Antibody titer was measured in plates coated with native TcdB, incubated with dilutions of sera, and detected with secondary antibody
- High-titer antibody response was observed following immunization with larger TcdB antigens

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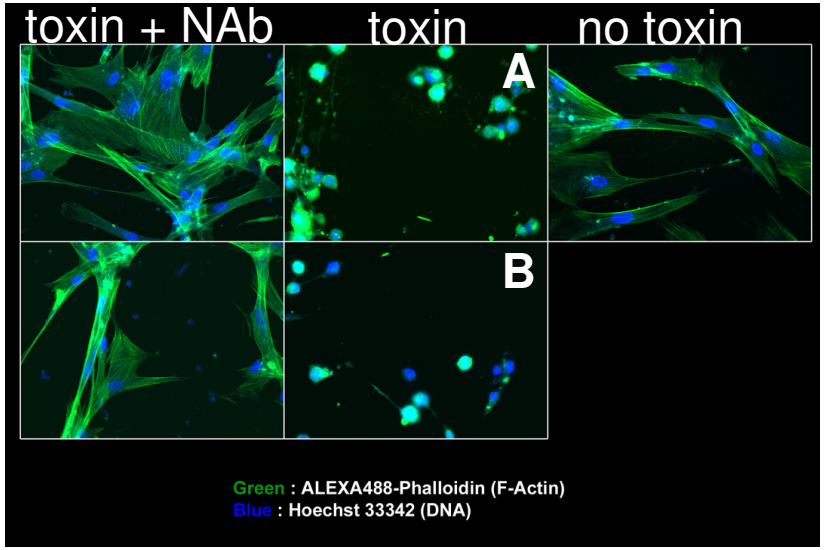
Neutralizing Activity of Antisera From Hamsters Immunized with TcdB Fragments

Assay Principle



Neutralizing Activity of Toxin B Antisera

TcdB fragment	Neutralizing activity
B1	++
B2	+
B3	+
B4	++
B0	+++
TMD	+++
TransCROPS	+++
Toxoid B	++++

- Native TcdB was incubated with tested antiserum for 1 hour and then added to IMR90 fibroblasts grown in 96-well microtiter plates. The extent of neutralization was assessed visually after 24-hour incubation
- Antisera collected from hamsters immunized with recombinant TcdB fragments effectively neutralized cytotoxic effects of native TcdB

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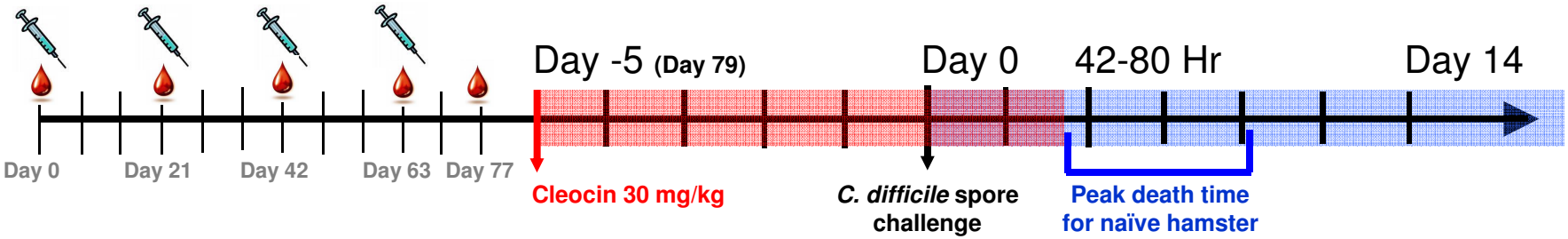
C. difficile Challenge in Syrian Hamsters – Experimental Outline



- Immunization phase
- Disruption & colonization phase
  - "Opening of a colonization window" that allows *C. difficile* to germinate and establish an infection
  - Requires antibiotic disruption of the gut microbiota
- Pathological phase
  - Colitis, typhilitis, wet tail
  - Ultimately death

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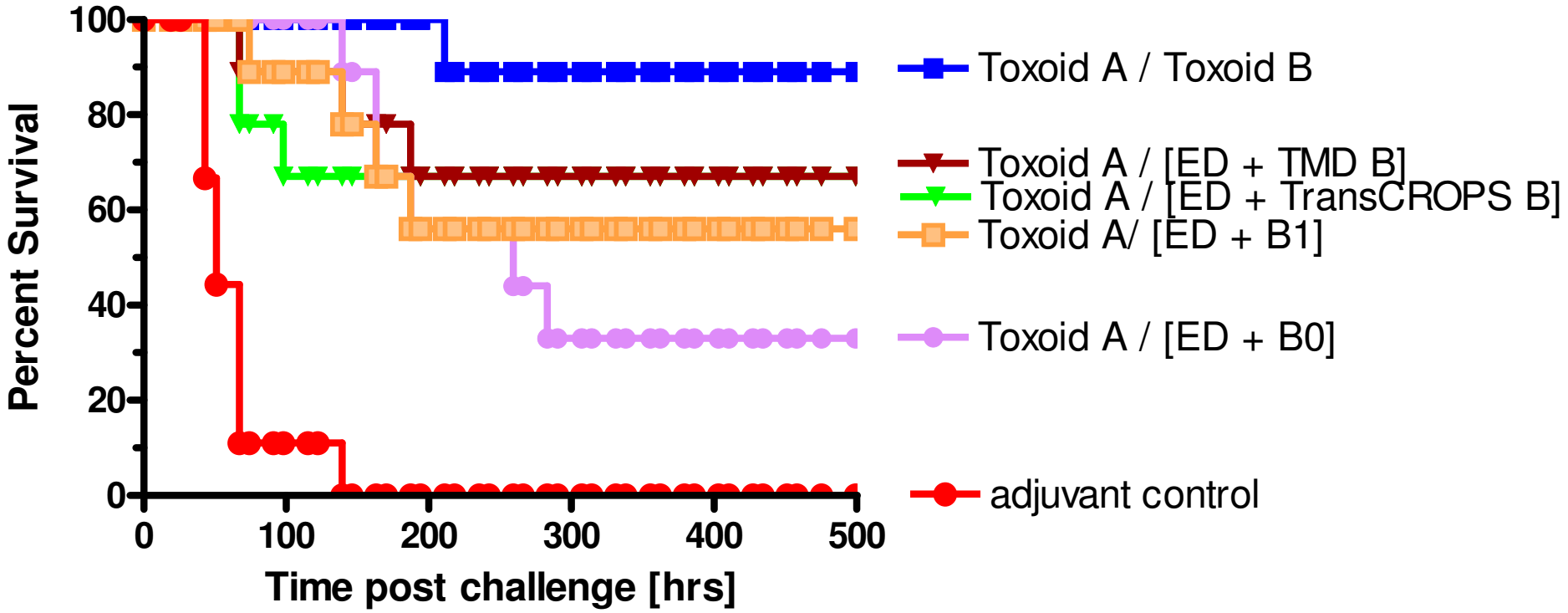
Protection of Hamsters in *C. difficile* Challenge Model



- Hamsters (n=10) immunized with a combination of native Toxoid A and recombinant fragments of TcdB were challenged with *C. difficile* spores
- Kaplan-Meier analysis demonstrated prolonged survival of hamsters immunized with recombinant antigens as compared with adjuvant control

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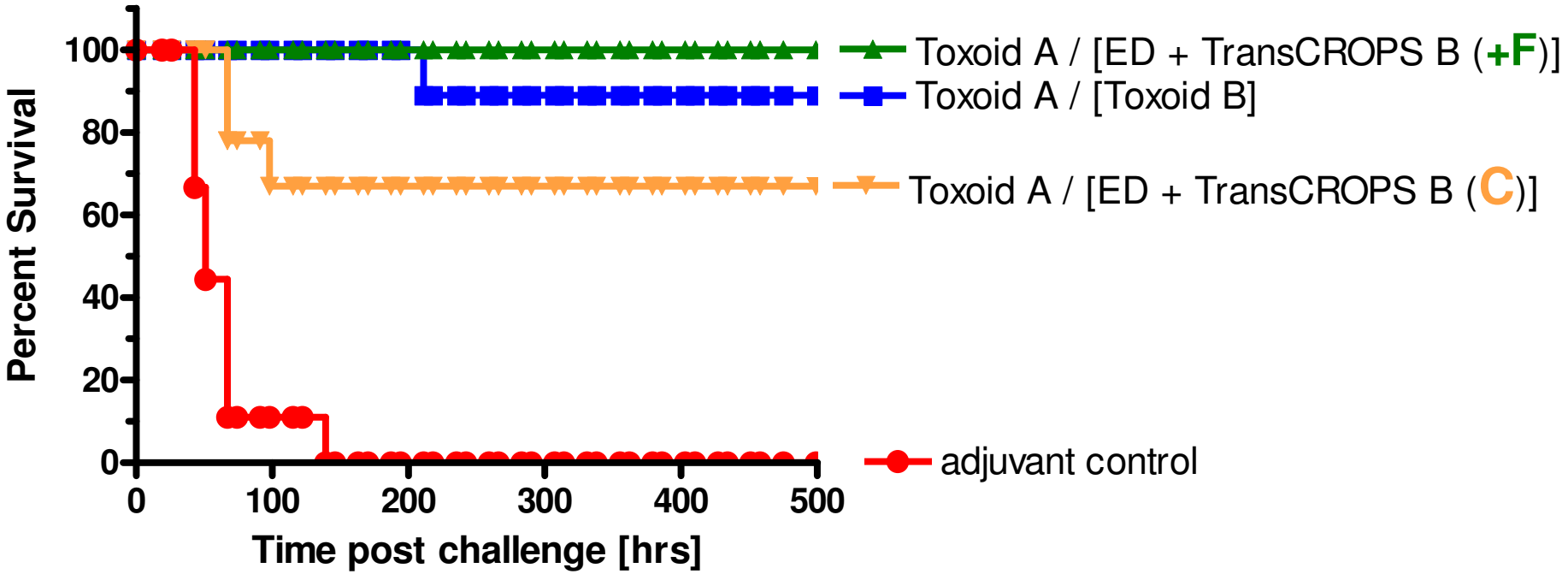
Protection of Hamsters in *C. difficile* Challenge Model – Effect of Formalin



- Hamsters (n=10) were immunized with a combination of native TcdA and ED+TransCROPS TcdB fragments formalin treated (+F) or untreated (C)

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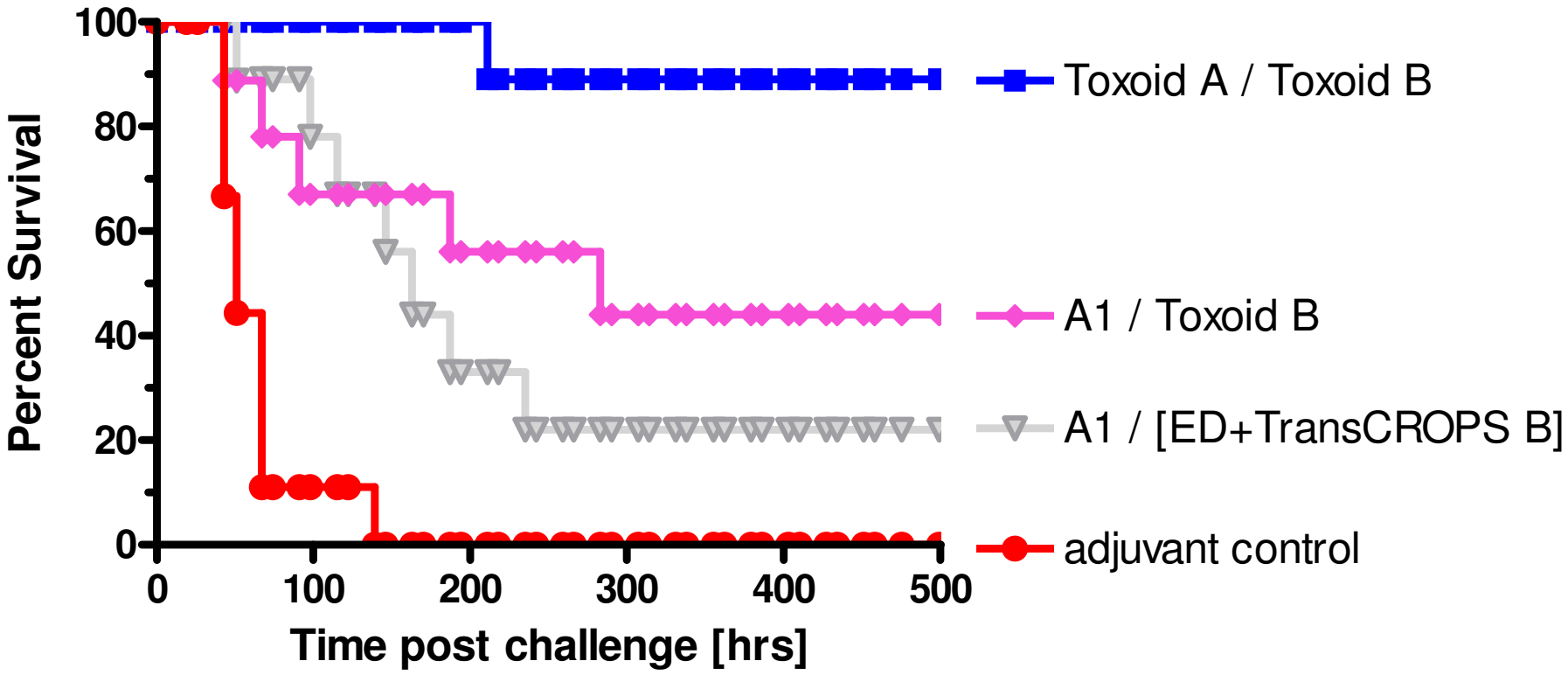
Protection of Hamsters in *C. difficile* Challenge Model – A1 Fragment of TcdA



- Hamsters (n=10) were immunized with a combination of either native or recombinant fragments of TcdB and recombinant fragment of TcdA (A1) and challenged with *C. difficile* spores

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Development of Full-Length Recombinant mTcdA and mTcdB Vaccine

- Full-length mTcdA (310 kDa) and mTcdB (270 KDa) were expressed using recombinant DNA technology
- Several mutations were introduced to reduce toxicity (4-5 log reduction achieved)
- Both antigens were purified to homogeneity in a fully scalable process

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Biophysical Analysis of Full-Length Recombinant mTcdA and mTcdB

				Circular dichroism (CD)		Melting temp	Analytical centrifugation (AUC)
Antigen	Source	α-helix	β-sheet	Turns	Unordered	Tm (°C)	kDa
mTcdA	**Recombinant**	**23**	**26**	**21**	**30**	**65**	**286 (by AUC)**
Toxin A	***C.difficile***	21	28	21	30		307 (Predicted)
Toxin A	Published*	32	23	22	22		307 (Predicted)
Toxoid A	Published*	32	25	19	24	59.8*	307 (Predicted)
mTcdB	**Recombinant**	**23**	**27**	**20**	**30**	**>85**	**240 (by AUC)**
Toxin B	***C.difficile***	21	28	21	29		270 (Predicted)
Toxin B	Published*	36	21	18	25		270 (Predicted)
Toxoid B	Published*	36	18	18	28	55.8*	270 (Predicted)

*J Pharm Sci. 2008 Oct;97(10):4194-207 (Acambis)

- CD spectra and analytical centrifugation revealed secondary structure and overall size comparable to native toxins and in good agreement with published analysis
- Recombinant mTcdA and mTcdB induced high ELISA titers and neutralizing antibodies (not shown)

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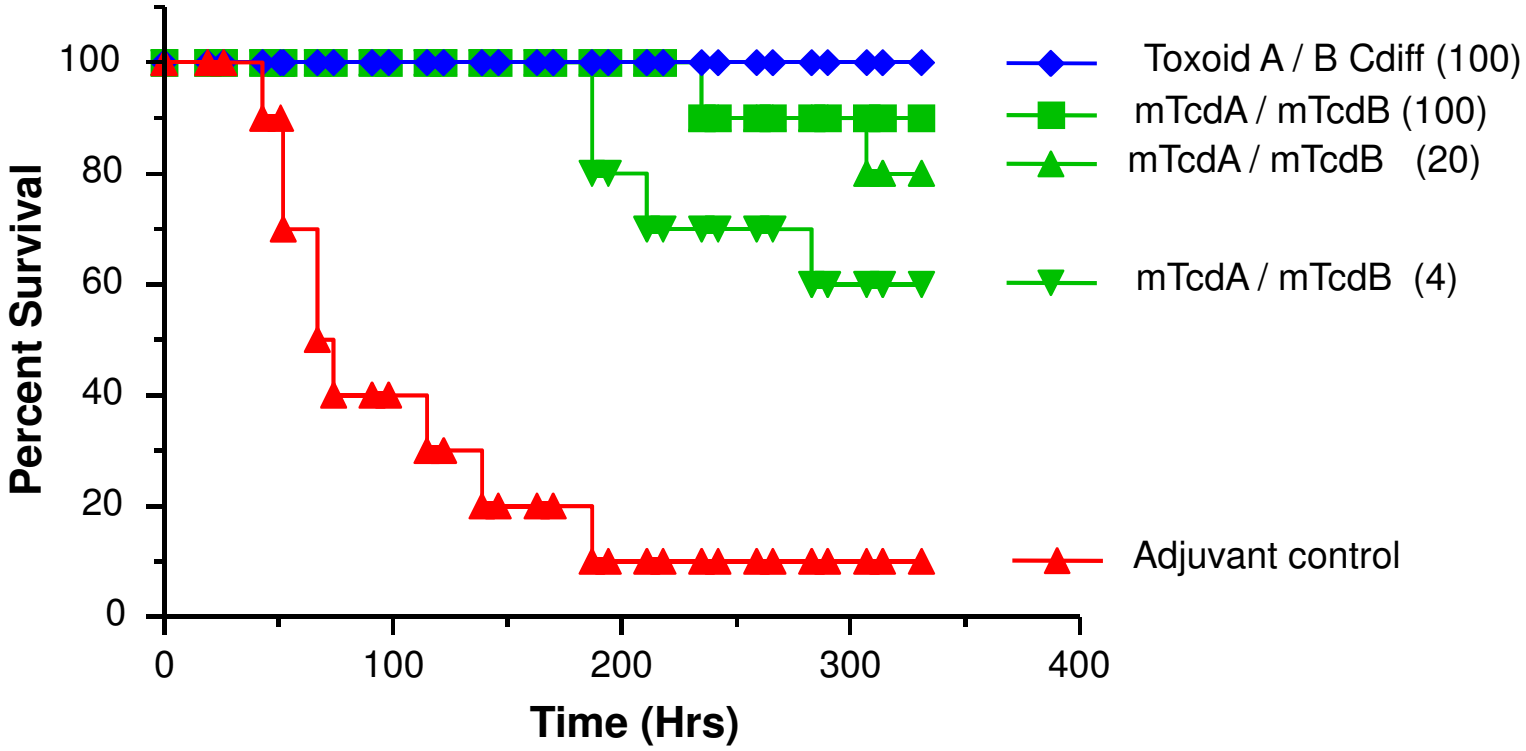
Efficacy of the Full-Length Recombinant mTcdA and mTcdB



- Recombinant TcdA/TcdB vaccine affords protection against *C. difficile* challenge (n=10)
- At the 100 µg dose (50 µg each) the level of protection is equivalent to benchmark toxoid vaccine
- Vaccine is also effective at 5-fold and 25-fold lower doses

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Summary

- Fragment-based vaccine consisting of the recombinant CROPS fragments of Toxin A and B induced high ELISA titers, neutralizing antibodies, and good protection against *C. difficile* challenge. Larger fragments were performing considerably better than small fragments of the CROPS region.

- Full-length recombinant mTcdA and mTcdB vaccine induced neutralizing antibodies and induced long-lasting protection equivalent to benchmark toxoid vaccine.

- The final vaccine image including formulation, stability, and dosing is under development.

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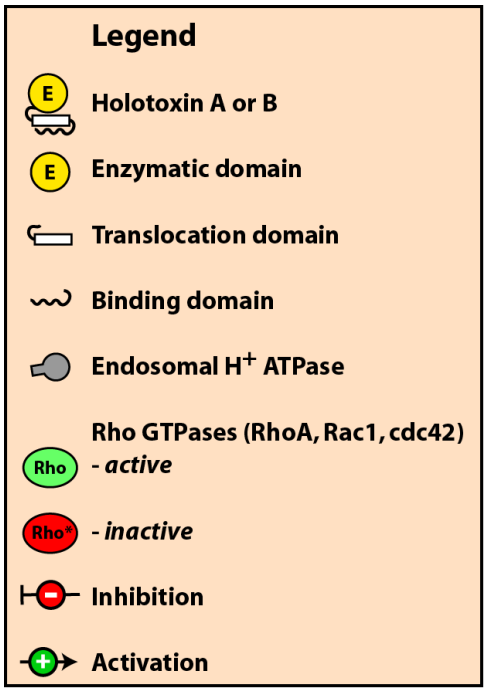
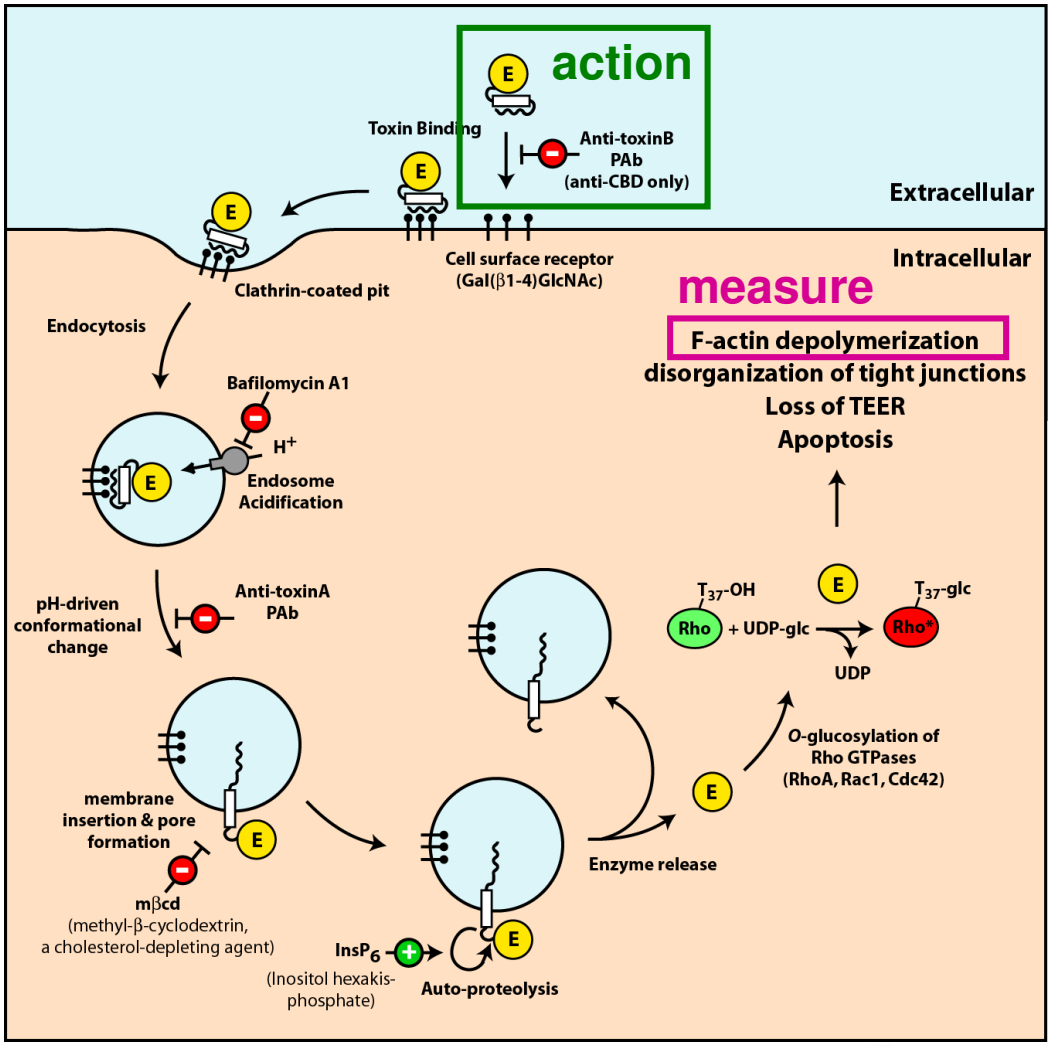
Cytotoxic Effects of *C. difficile* Toxins





Barth et al, JBC 276(2001):10670-10676
Gieseman et al, JBC 281(2006):10808-10815
Rupnik et al, Microbiology 151(2005):199-208
Pfeifer et al, JBC 278(2003):44535-44541

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Abstract

Clostridium difficile is the major cause of antibiotic-associated diarrhea and pseudomembranous colitis, a disease associated with significant morbidity and mortality. The disease is mostly of nosocomial origin, with elder patients undergoing anti-microbial therapy being particularly at risk. A new, hypervirulent strain of *C. difficile* called NAP1 (027) has been implicated in outbreaks associated with increased morbidity and mortality since the early 2000s. This epidemic strain is responsible for increased incidence of *C. difficile*-associated diarrhea related not only to antibiotic exposure, but infection is also associated with GI surgery, prolonged hospitalization, and immune-compromising conditions. *C. difficile* produces 2 large toxins: Toxin A and Toxin B. These 2 toxins act synergistically to damage and impair the colonic epithelium and are primarily responsible for the pathogenesis associated with *C. difficile* infection (CDI). Testing the feasibility of toxin-based vaccination against *C. difficile* is being investigated. A native Toxoid A- and B-based vaccine was reported to be safe and immunogenic in healthy volunteers.

We generated a toolbox of *E. coli* expressed Toxin B fragments covering the entire molecule and systematically explored these fragments as components of an experimental vaccine. We observed a robust immune response in hamsters vaccinated with the recombinant toxin fragments. The antiserum obtained from immunized hamsters was shown to neutralize cytotoxic effects of Toxin B *in vitro* (in cell-based neutralization assay). Hamsters immunized with the combination of full-length Toxoid A and fragments of Toxin B were protected against lethal challenge with *C. difficile* spores.

We also evaluated that recombinant full-length mTcdA and mTcdB vaccine and demonstrated excellent performance in a hamster challenge model, equivalent to benchmark toxoid vaccine.

The use of recombinant *C. difficile* toxins could afford improved protection, vaccine thermal stability, and facilitate manufacturability of the vaccine. The additional studies, including thermal stability, formulation, safety, and dosing, will be needed to establish the final vaccine image.

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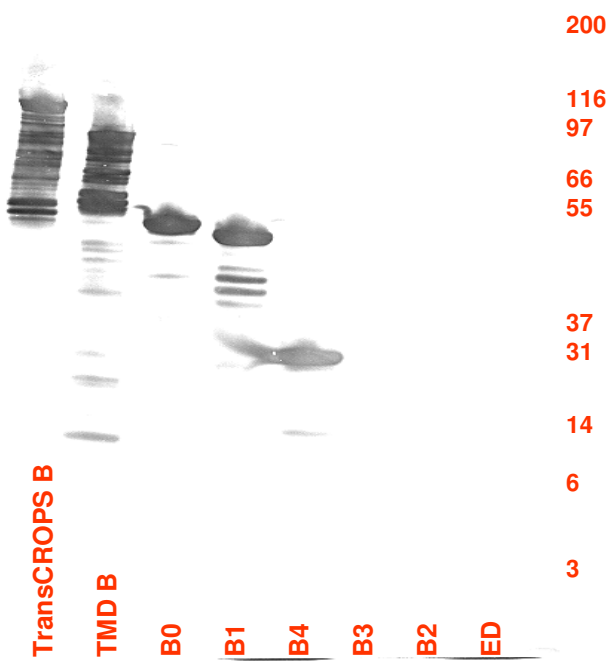
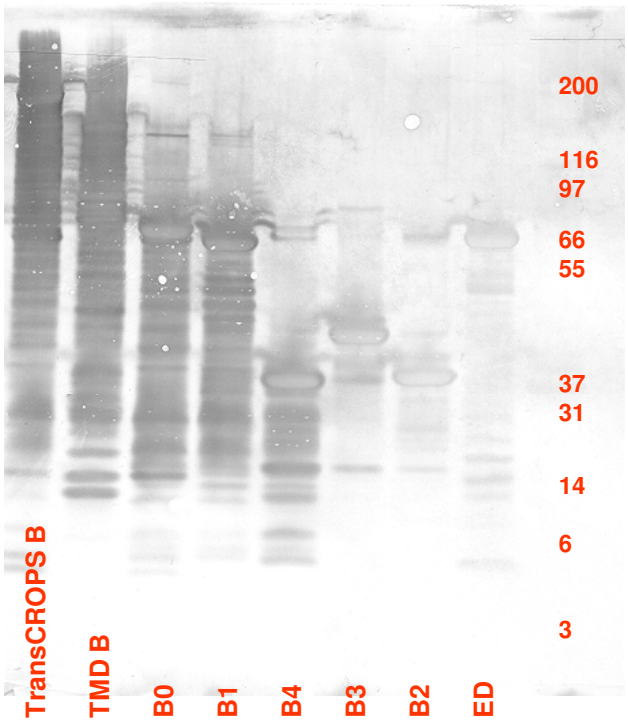
Western Blotting Analysis of Recombinant Toxin B Fragments

A. Monoclonal antiTcdB (Novus)



B. Polyclonal antiTcdB/TcdA



- Recombinant Toxin B fragments were separated on SDS/PAGE, transferred onto nitrocellulose, and probed with monoclonal antobody (5A8-E11) developed against full-length native Toxin B (Novus Biologicals) (A) or polyclonal antisera from rhesus monkeys immunized with Toxin A/Toxin B (B)

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C. difficile Infection Prevention: Biotherapeutics, Immunologics, and Vaccines

Table 2. Attributes of Potential Strategies for Prevention of *C. difficile* Infection.

Prevention Intervention	Effectiveness in Humans	Rapidity of Prevention	Duration of Prevention	Primary Prevention	Recurrence Prevention	Projected Cost
Fecal trans-plants	Excellent	Rapid (1-2 days)	Likely to be effective until further antibiotics	Unknown	Yes	Low
Non-toxigenic *C. difficile*	Unknown but expected to be excellent from natural colonization	Rapid (1-2 days)	Likely to be effective until microbiota recovers or further antibiotics	Yes	Yes	Low
Injectable vaccine	Unknown	Slow (weeks to months)	Unknown but should be long	Yes	Yes	Low
Mucosal vaccine	Unknown	Slow (weeks to months)	Unknown but should be long	Yes	Yes	Low
Monoclonal antibodies	Excellent	Rapid (immediate)	Unknown but expected to be transient	Probable	Yes	Very high

Discov Med. 2012 Jan;13(68):75-83.
Gerding DN

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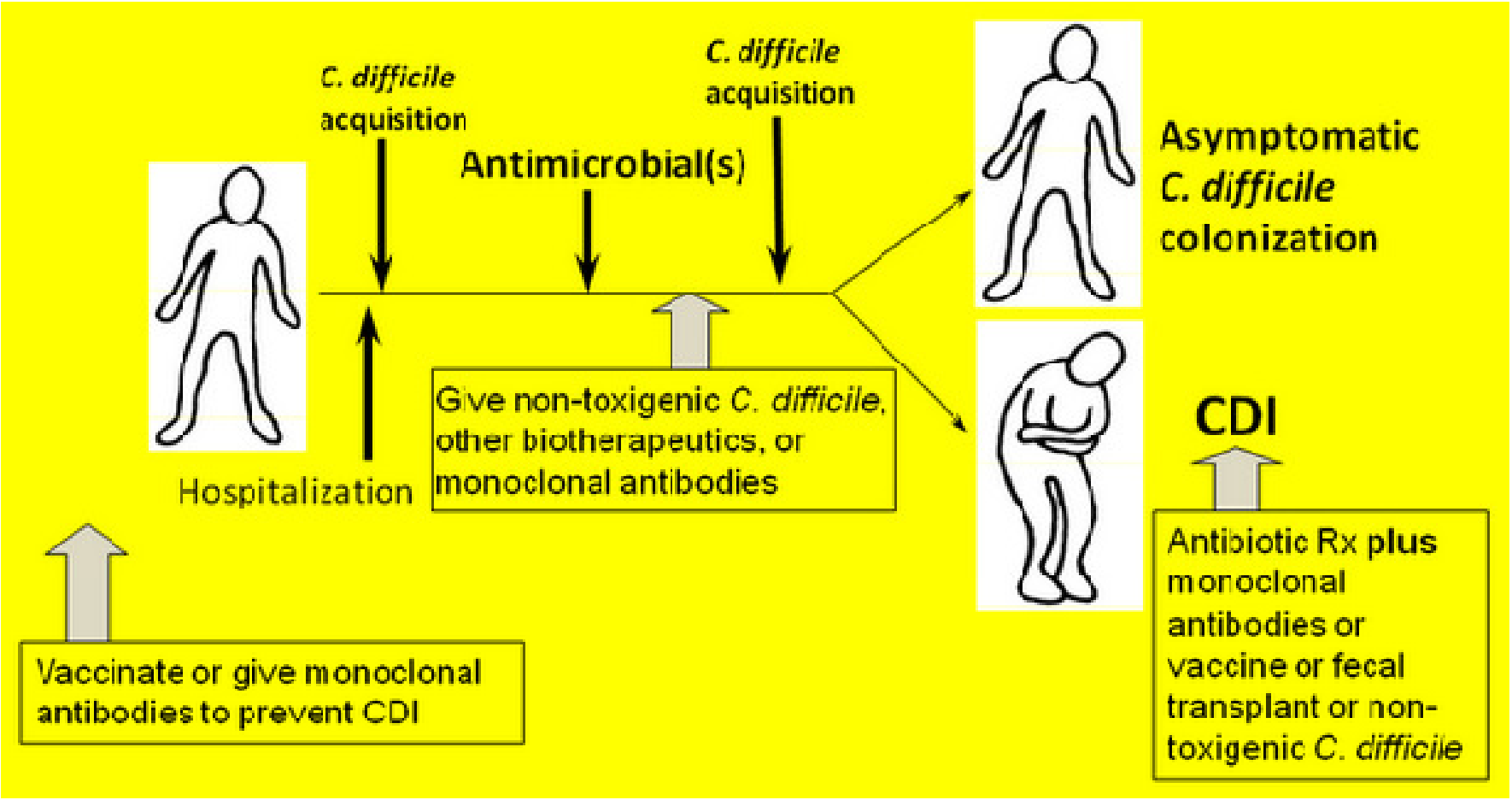
Points of Treatment of CDI



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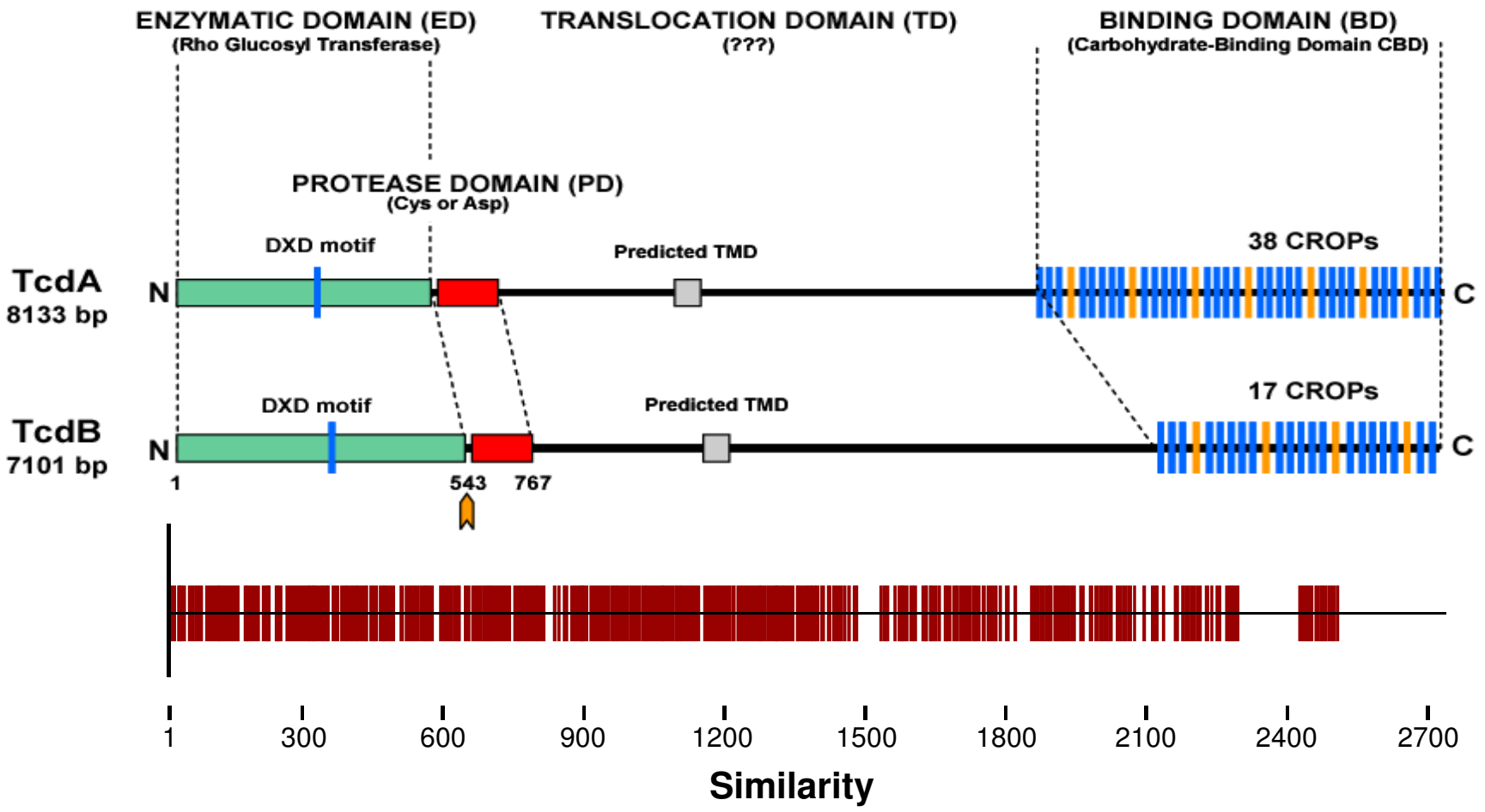
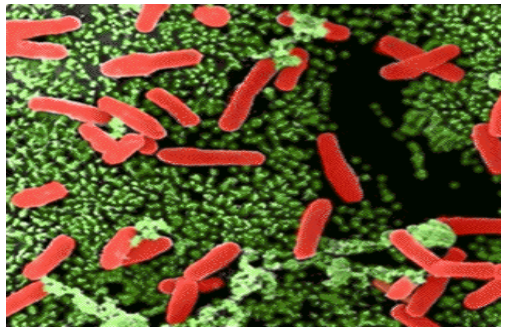
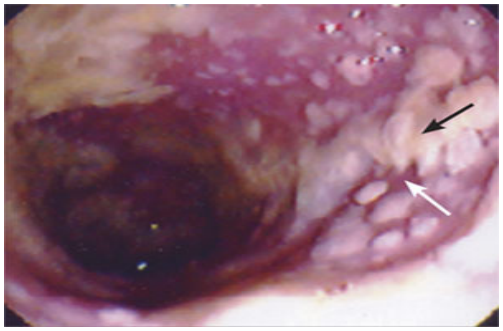
Sequence Similarity Between TcdA and TcdB

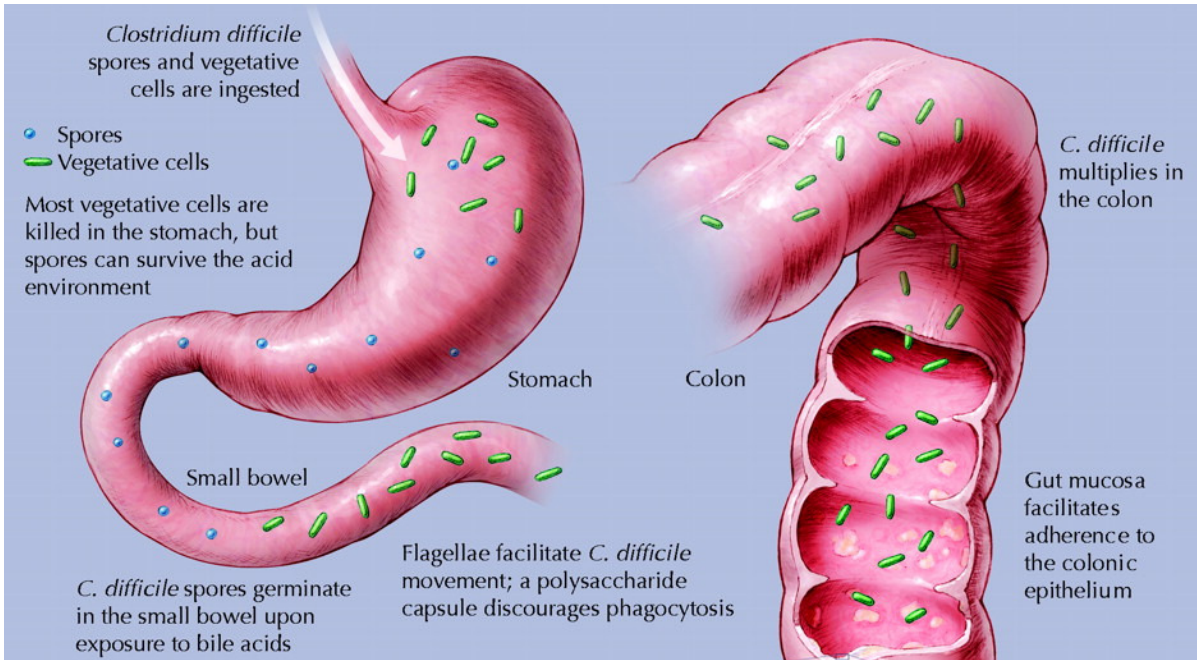


C. difficile Infection

- 500,000 hospitalizations in US due to CDI – up to 5% mortality
- Anaerobic spore-forming gram-positive bacillus
- Causes *C. difficile*-associated infection (CDI or CDAD)
- Damage to gut lining → diarrhea → pseudomembranous colitis → toxic megacolon → sepsis → death
- Main mode of transmission is fecal-oral in hospital setting







2011 symptomsbook.com

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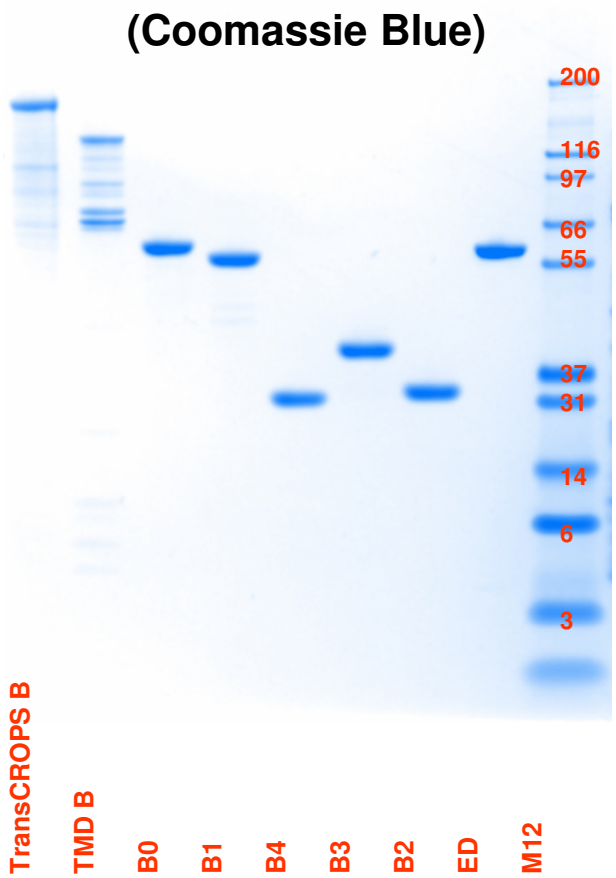
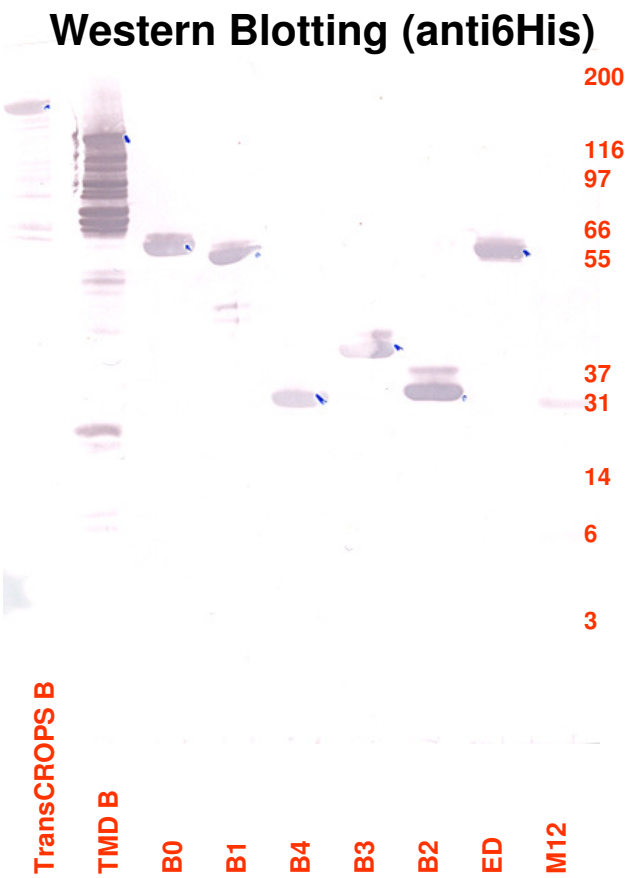
Expression and Purification of Recombinant Fragments of TcdB





- Synthetic genes encoding TcdB fragments were cloned into pET30a vector, expressed in (BL21/DE3) *E. coli,* and purified by two-step chromatography using Ni-affinity column followed by anion exchange chromatography

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Expression of TcdB Fragments in *E. coli* – Purification Yields

Fragment Name	Amino Acids	Mw	# of Residues	pI	# of Cys	Purity, %	Yield* mg/Liter
TransCROPS B	545-2367	207	1832	4.37	8	**77**	**39**
TMD B	1129-2367	142	1248	4.3	4	**~50**	**50**
B0	1786-2367	68	592	4.11	1	**95**	**822**
B1	1836-2367	62.4	542	4.13	1	**99.3**	**582**
B2	1836-2101	31.6	275	4.4	0	**99.3**	**1836**
B3	1949-2275	39.1	336	4.29	1	**99.6**	**686**
B4	2102-2367	32	276	4.07	1	**99.6**	**800**
ED B		63.9	559	4.85	1	**91**	**505**

* Assuming 20% biomass, after two-step purification

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