New Redesign of Recombinant Attenuated *Salmonella* Vaccines

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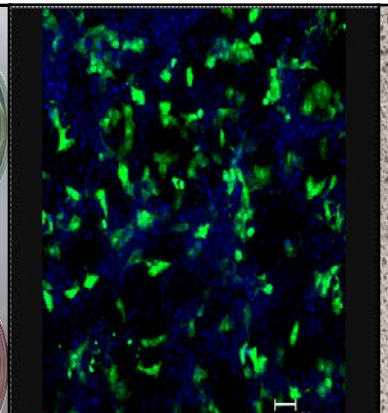


Objectives for Orally Delivered Live *Salmonella* Vectored Vaccines

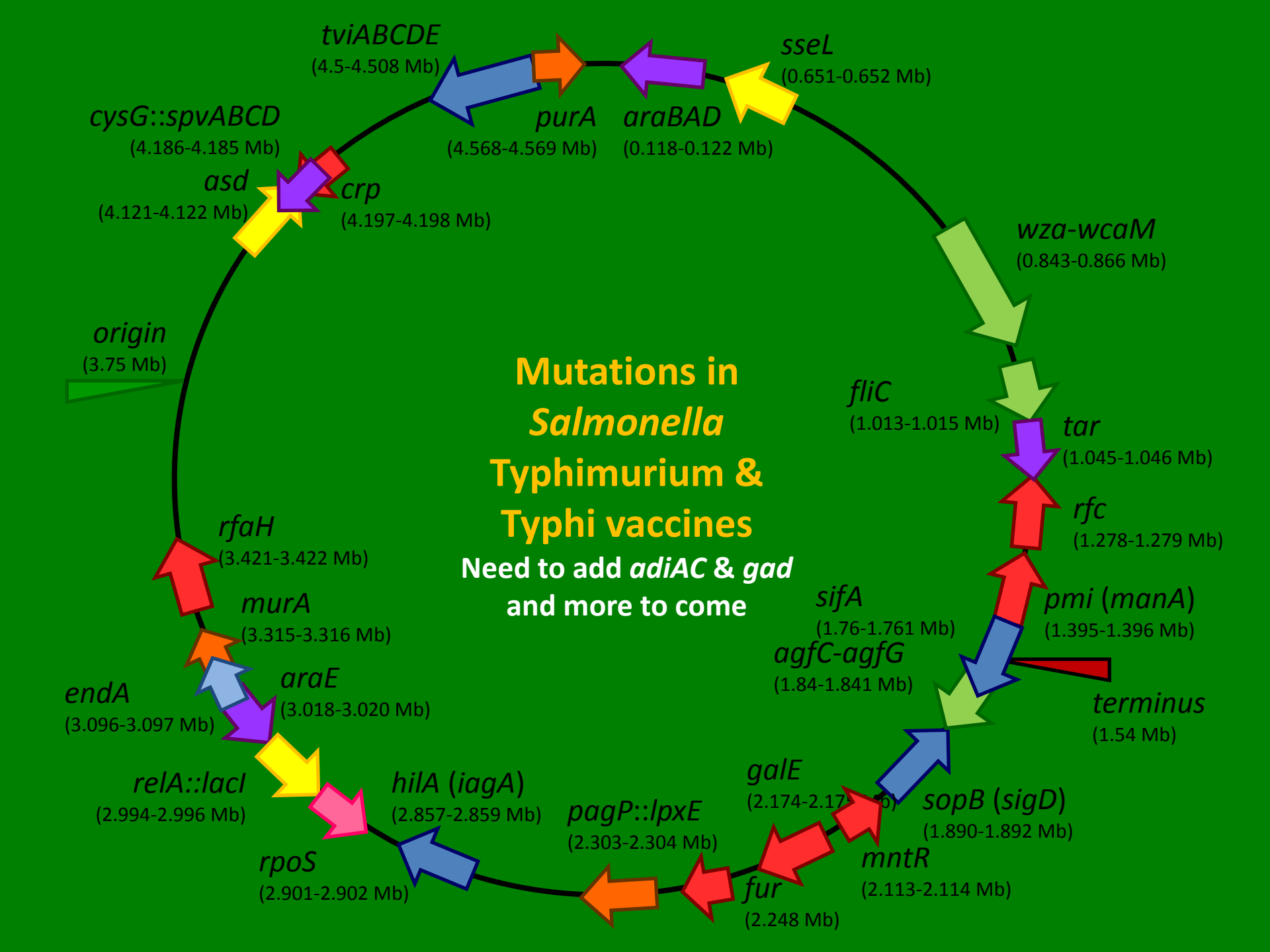
- Maximize invasiveness to and colonization of internal effector lymphoid tissues
- Recruit innate immune responses without being over reactogenic to cause distress or disease symptoms
- Maximize induction of mucosal, systemic and cellular immunities
- Be safe for newborn, pregnant, malnourished and immunocompromised individuals
- Exhibit biological containment

Why *Salmonella* as a vaccine vector?

Mucosally-delivered *Salmonella enterica* Serotypes efficiently attach to and invade mucosal associated lymphoid tissues (MALT, GALT, NALT, BALT, etc.) in mice and humans before colonizing internal lymph nodes, liver and spleen. They can also be delivered parenterally.

All routes of delivery result in induction of mucosal, systemic and cellular immune responses with induction of long-term protective immunity. Attenuated pathogens that are not invasive or only invasive to mucosal tissues seldom induce long-lasting immunity.

We know a great deal about the pathogenicity, physiology, genetics and genomics of *Salmonella* serotypes and *Salmonella* can be genetically manipulated with ease.


Mutations in
Salmonella
Typhimurium &
Typhi vaccines
Need to add adiAC & gad
and more to come
tviABCDE
(4.5-4.508 Mb)
sseL
(0.651-0.652 Mb)
cysG::spvABCD
(4.186-4.185 Mb)
purA
(4.568-4.569 Mb)
araBAD
(0.118-0.122 Mb)
asd
(4.121-4.122 Mb)
crp
(4.197-4.198 Mb)
wza-wcaM
(0.843-0.866 Mb)
origin
(3.75 Mb)
fliC
(1.013-1.015 Mb)
tar
(1.045-1.046 Mb)
rfc
(1.278-1.279 Mb)
rfaH
(3.421-3.422 Mb)
sifA
(1.76-1.761 Mb)
pmi (manA)
(1.395-1.396 Mb)
murA
(3.315-3.316 Mb)
agfC-agfG
(1.84-1.841 Mb)
endA
(3.096-3.097 Mb)
araE
(3.018-3.020 Mb)
terminus
(1.54 Mb)
relA::lacI
(2.994-2.996 Mb)
hilA (iagA)
(2.857-2.859 Mb)
galE
sopB (sigD)
(1.890-1.892 Mb)
pagP::lpxE
(2.303-2.304 Mb)
rpoS
(2.901-2.902 Mb)
mntR
(2.113-2.114 Mb)
fur
(2.248 Mb)

Live Attenuated Bacterial Platforms

Each of three platforms use many newly developed innovative means based on extensive genetic modification to yield vectors:

1. To deliver proteins (antigens) by T2SS, T3SS and/or T5SS to endosome or cytosol of host cells without lysing
2. To deliver proteins (antigens) by regulated delayed lysis in the endosome or cytosol of host cells (can also include delivery prior to lysis by using T2SS, T3SS and T5SS)
3. To deliver DNA vaccine vectors encoding proteins (antigens) by regulated delayed lysis in cytosol of host cells to be synthesized and post-translationally modified in the immunized animal or human host

All strains are sensitive to all antibiotics.

We achieve our objectives by genetically engineering *Salmonella* to exhibit:

1. Regulated delayed in vivo expression of attenuating phenotype

2. Regulated delayed in vivo expression of codon-optimized sequences encoding protective antigens to be delivered by Type 2, 3 or 5 secretion systems and/or by regulated delayed lysis in vivo

3. Expression of arginine decarboxylase and/or glutamate decarboxylase at time of vaccine administration to neutralize stomach acidity to enable more vaccine cells to reach the ileum

These three features enable the vaccine strain at the time of oral vaccination to exhibit the same or better attributes than the wild-type parental strain to survive host-induced stresses to increase ability to colonize internal lymphoid tissues to maximize immunogenicity.

We achieve our objectives by genetically engineering *Salmonella* to exhibit (cont'd):

4. Constitutive expression of a highly invasive phenotype to enhance colonization of lymphoid tissues to induce maximal levels of protective immunity

This decreases dose of vaccine needed to induce protective immunity

5. Decreased ability to induce antibody responses to serotype-specific and immuno-dominant surface antigens

This facilitates reuse of the vaccine vector for multiple vaccines

6. Decreased ability to suppress or modulate induction of immunity

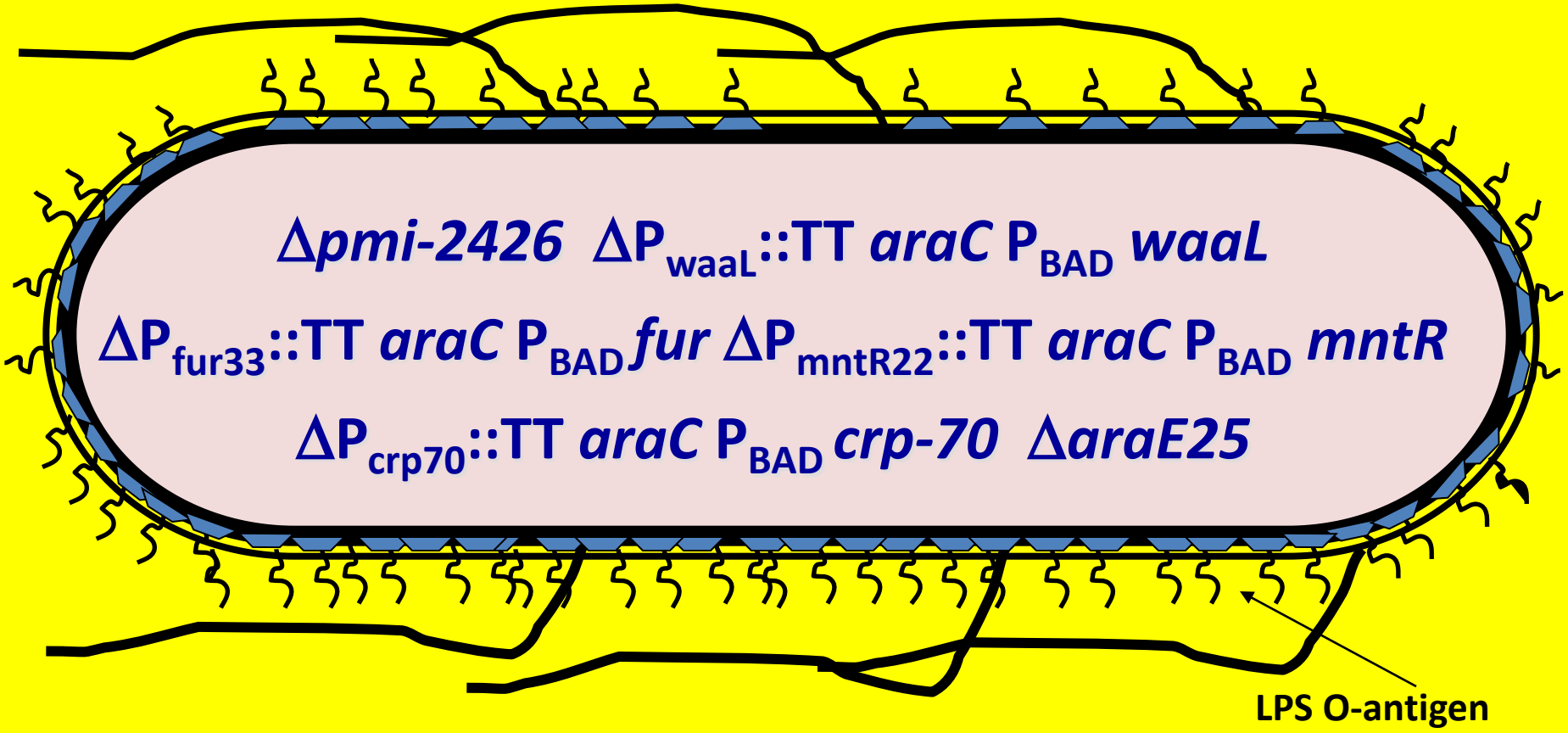
These multiple alterations also act to increase induction of mucosal immunity, decrease inflammation of the intestinal tract and decrease ability to form biofilms and thus lessen persistence of vaccine cells in vivo

Regulated delayed in vivo expression of the attenuating phenotype is achieved by:

1. Turning off ability to synthesize LPS O-antigen (and/or parts of the LPS core) in vivo (5 means).
2. Replacing promoters for genes needed to display virulence with regulatable promoters (2 types) that will be on for vaccine strain growth and initial colonization of the GI tract and lymphoid tissues and then be turned off in vivo to preclude disease symptoms (8 means).
3. Using regulated delayed lysis in vivo as the ultimate means of attenuation with complete biological containment and no shedding of survivors (1 basic strategy with multiple modifications to alter timing).

- **<u>One of our objectives</u> is to construct a safe, efficacious *Salmonella* vaccine vector system that will enable repetitive use of the vector system to develop multiple Recombinant Attenuated *Salmonella* Vaccines (RASVs) against a diversity of animal pathogens, yet would induce cross-protective immunity to diverse *S. enterica* serotypes and other pathogenic enteric bacteria colonizing chickens, other farm animals and humans.**

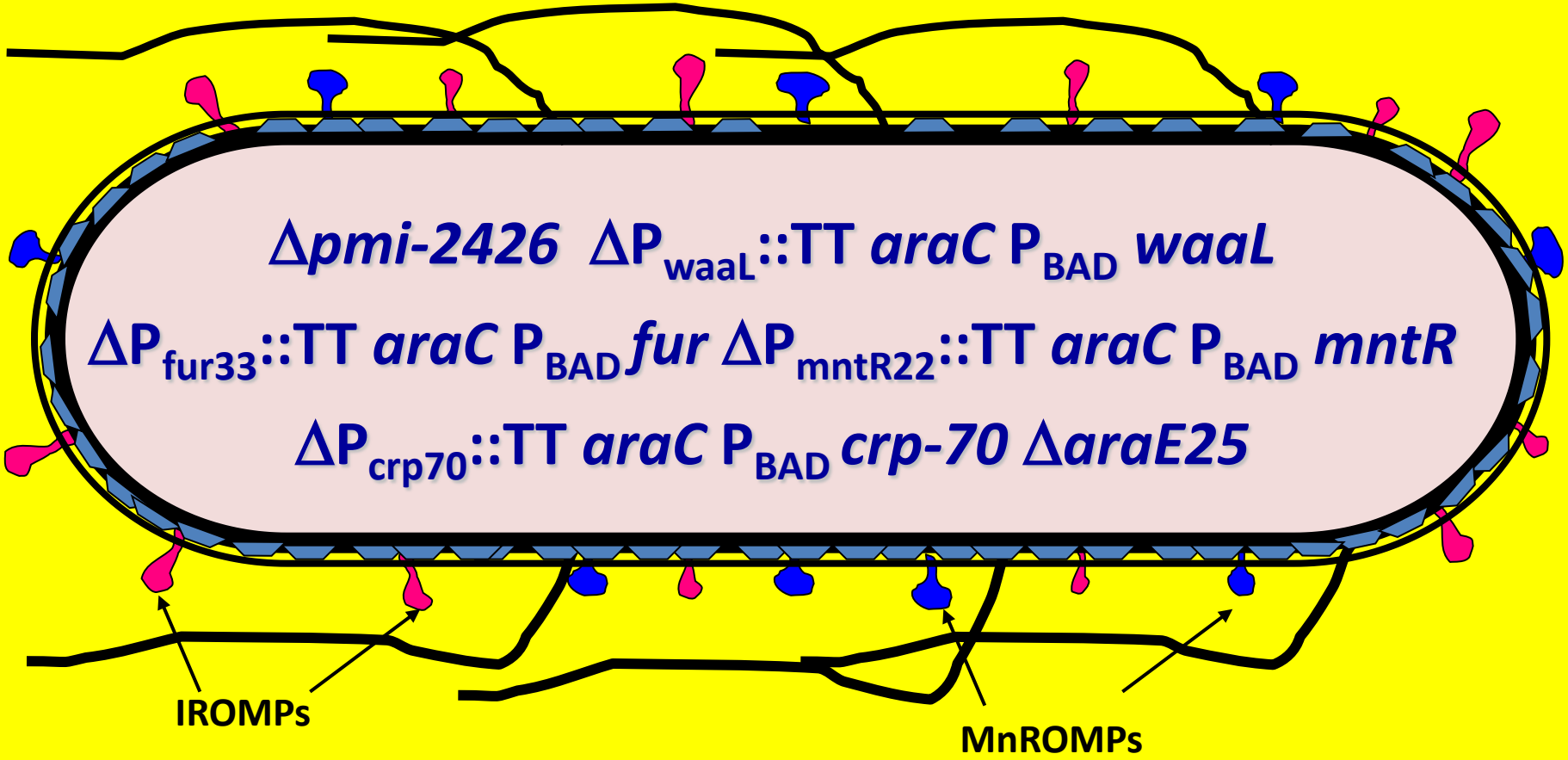
A. Vaccine strain at time of vaccination



= LPS core

No IROMP or MnROMP expression

B. Vaccine strain in lymphoid tissues

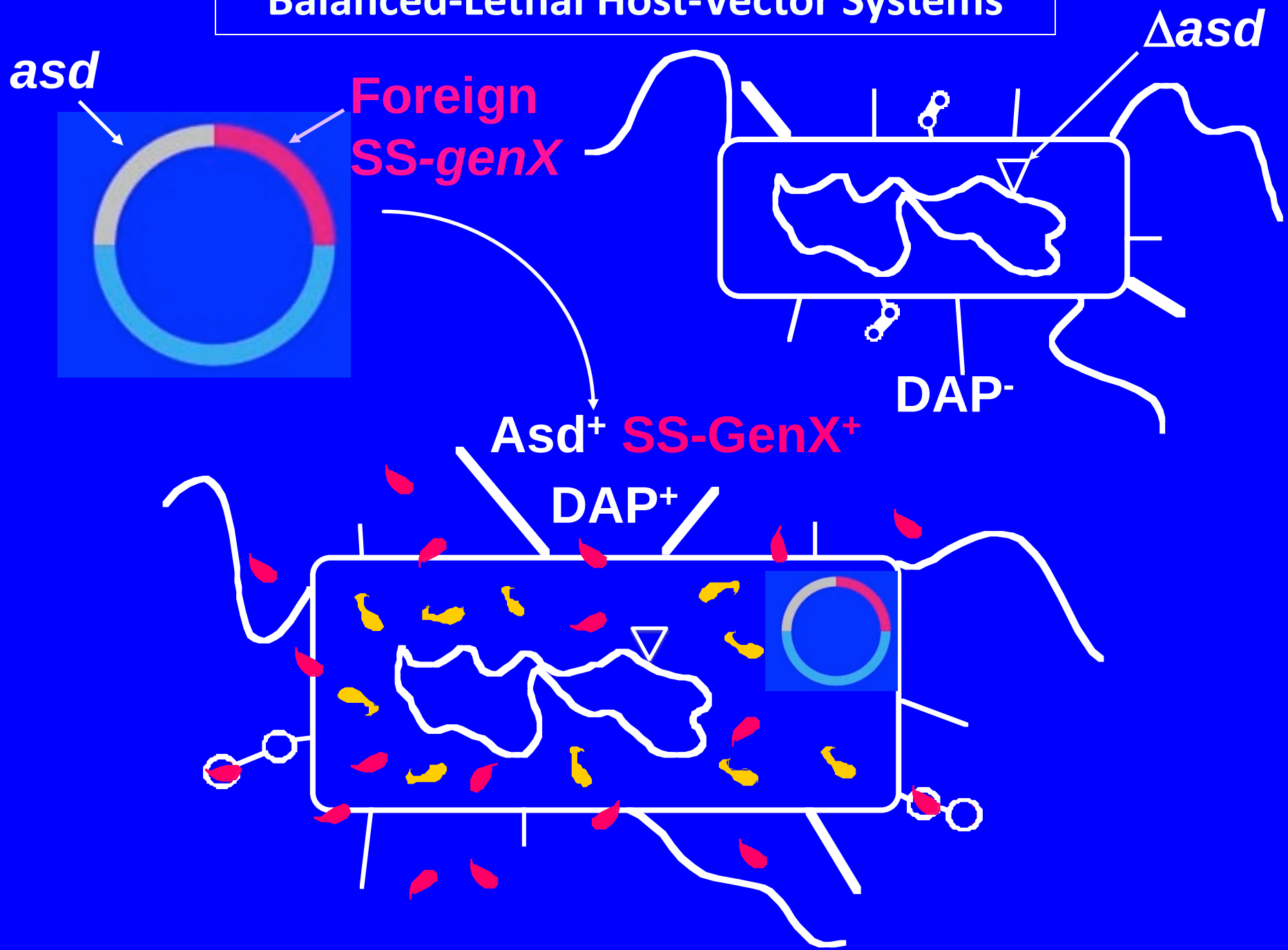


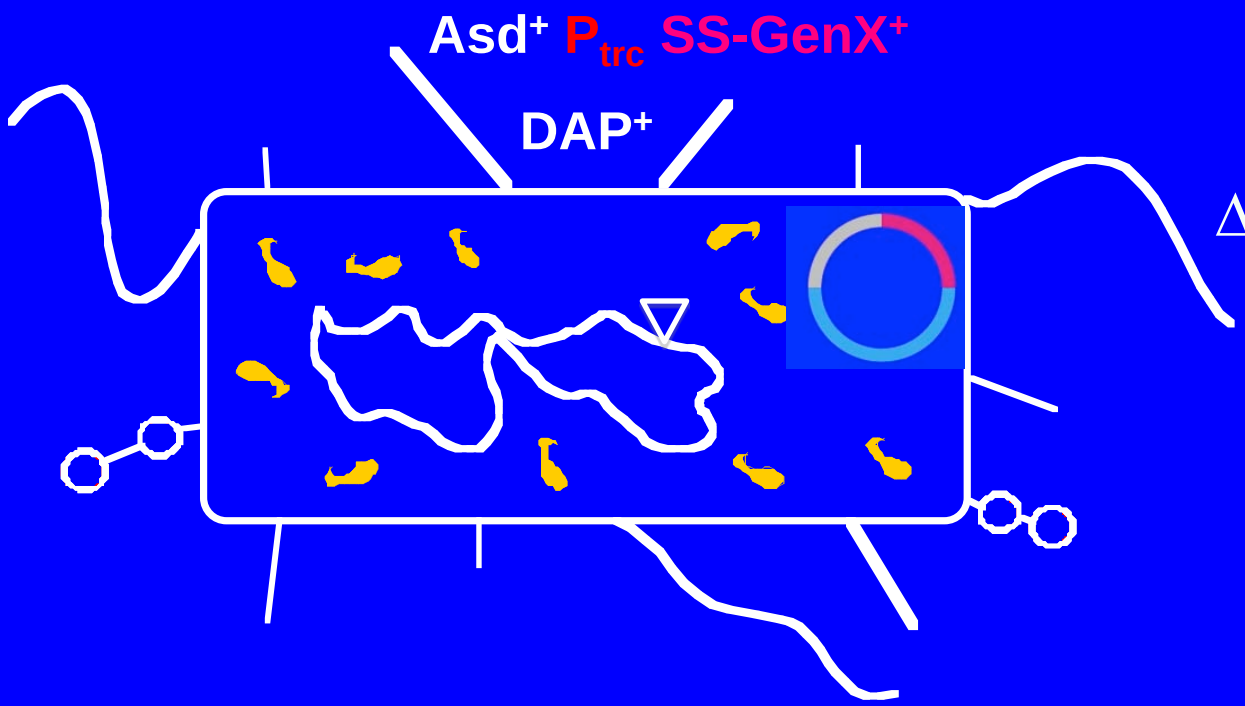
= LPS core

No LPS O-antigen

Expression of protective IROMPs, MnROMPs and LPS core

Balanced-Lethal Host-Vector Systems



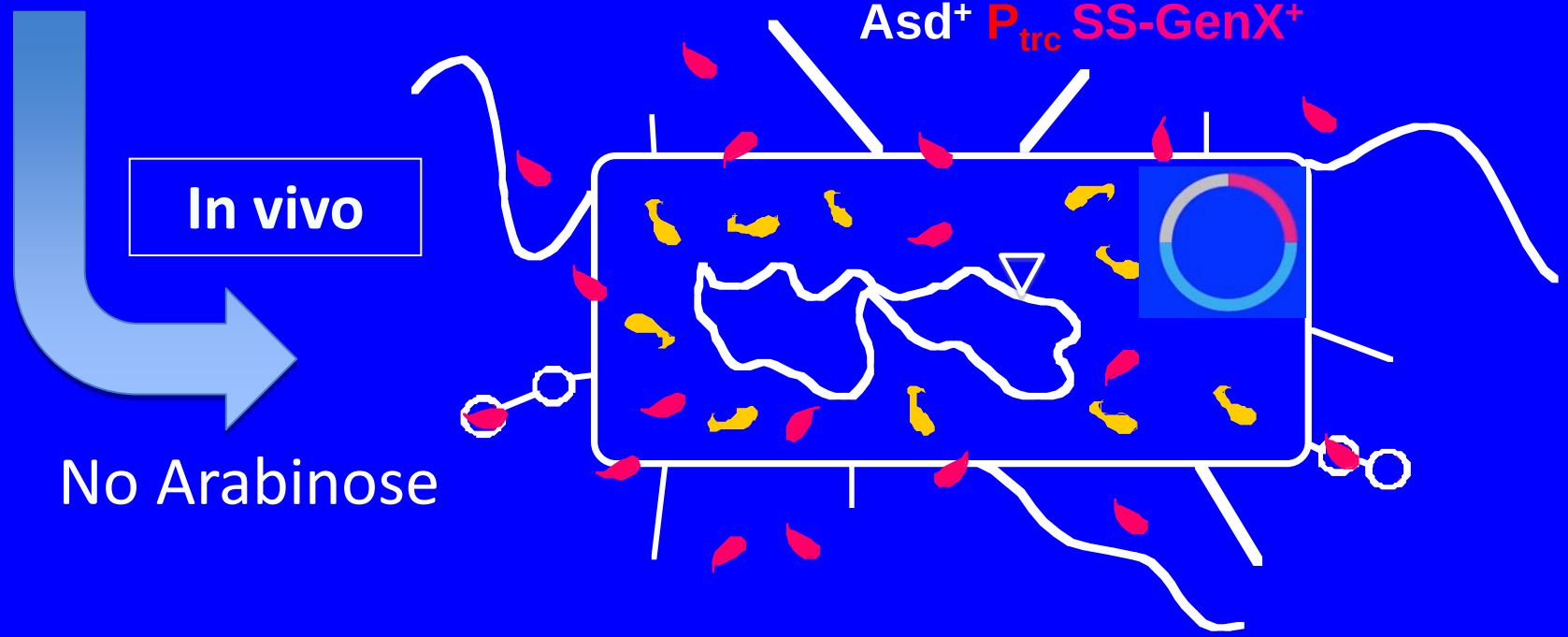


In *Salmonella*

Δ*relA*::*araC* P_{BAD} *lacI* TT

+ Arabinose

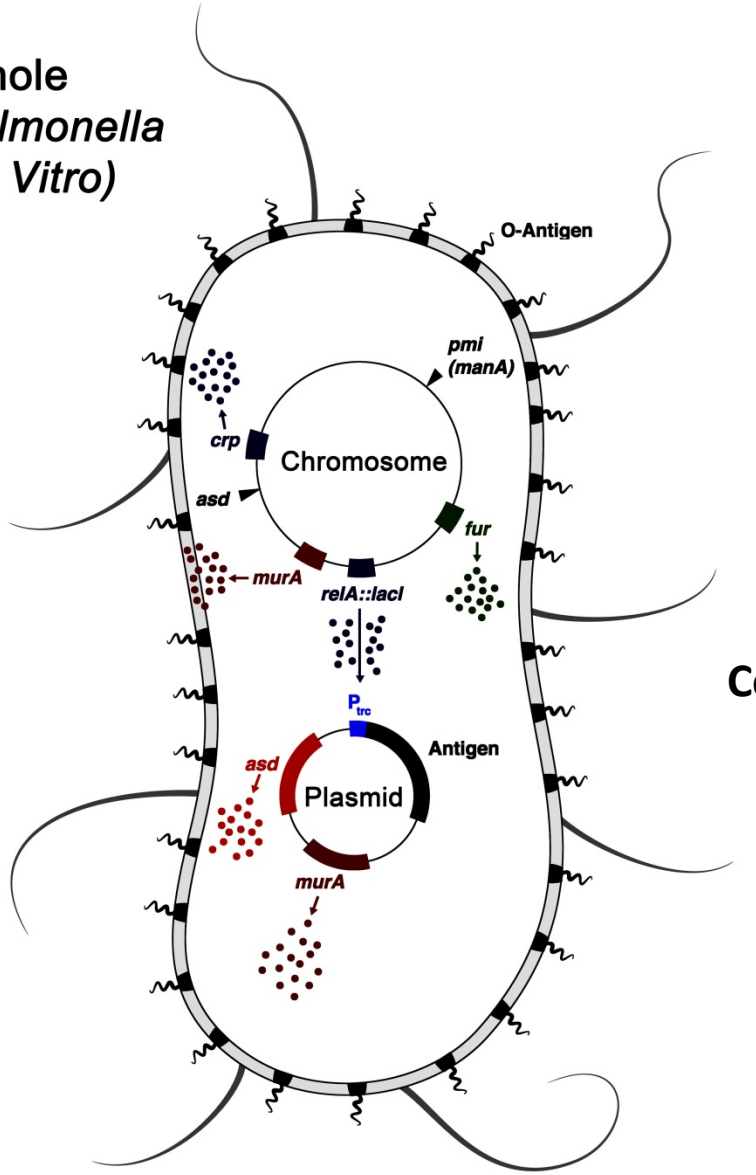
produce LacI



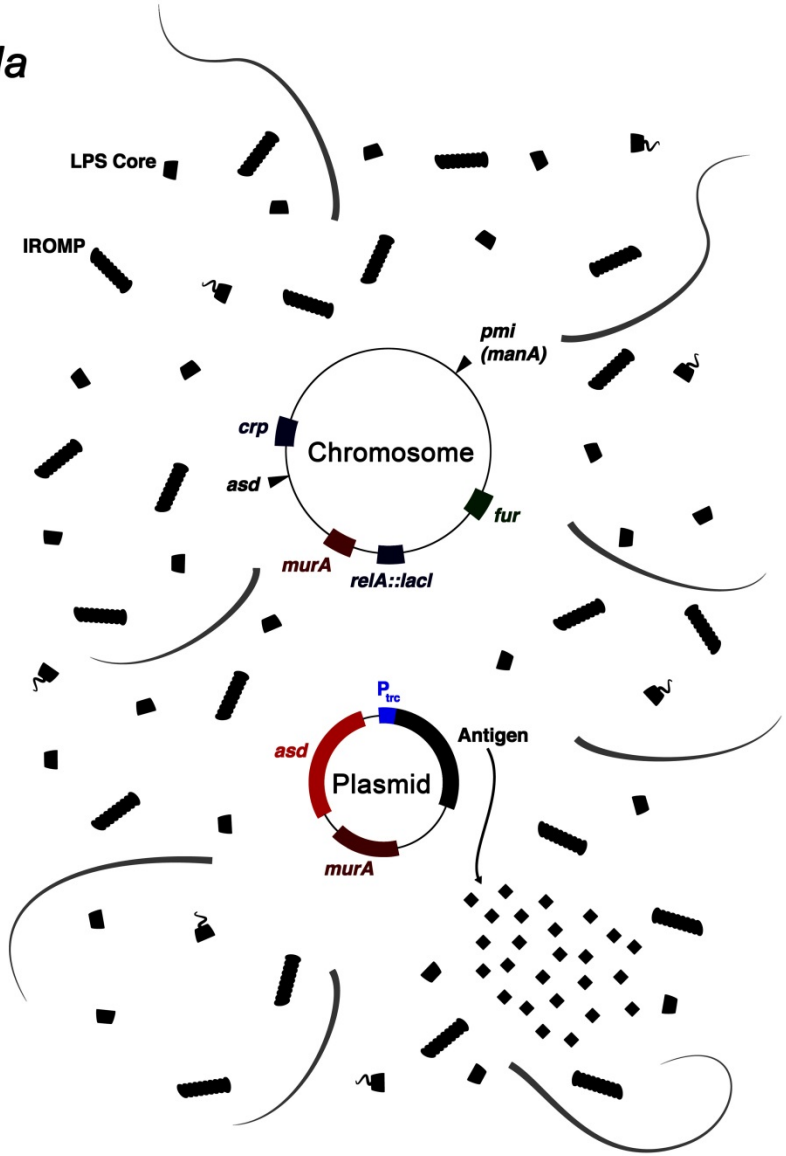
Regulated Delayed in vivo Lysis of Vaccine Strain

Achieved by regulated expression of essential genes for synthesis of muramic acid and diaminopimelic acid (DAP) in peptidoglycan

- Is a type of regulated delayed attenuation
- Results in no vaccine strain persistence in vivo and no survivors if excreted
- Can be used to deliver a bolus of protective antigen in host cell endosome or cytosol or a DNA vaccine vector in cytosol



Cell division ⇒



Decreasing: Fur, MurA, Crp, LacI, Asd, O-Antigen

Increasing: Protective Antigen Production

Food Safety

A high proportion of all enteric viral and bacterial infectious diseases that result in 3 million global annual deaths are transmitted through the food chain either due to the presence of these pathogens in or on animal and plant foods or by contamination of these foods with water containing these enteric pathogens. Although farm animals have been implicated as the source of these enteropathogens it is now clear that vegetables, fruits and humans are also vectors of transmission.

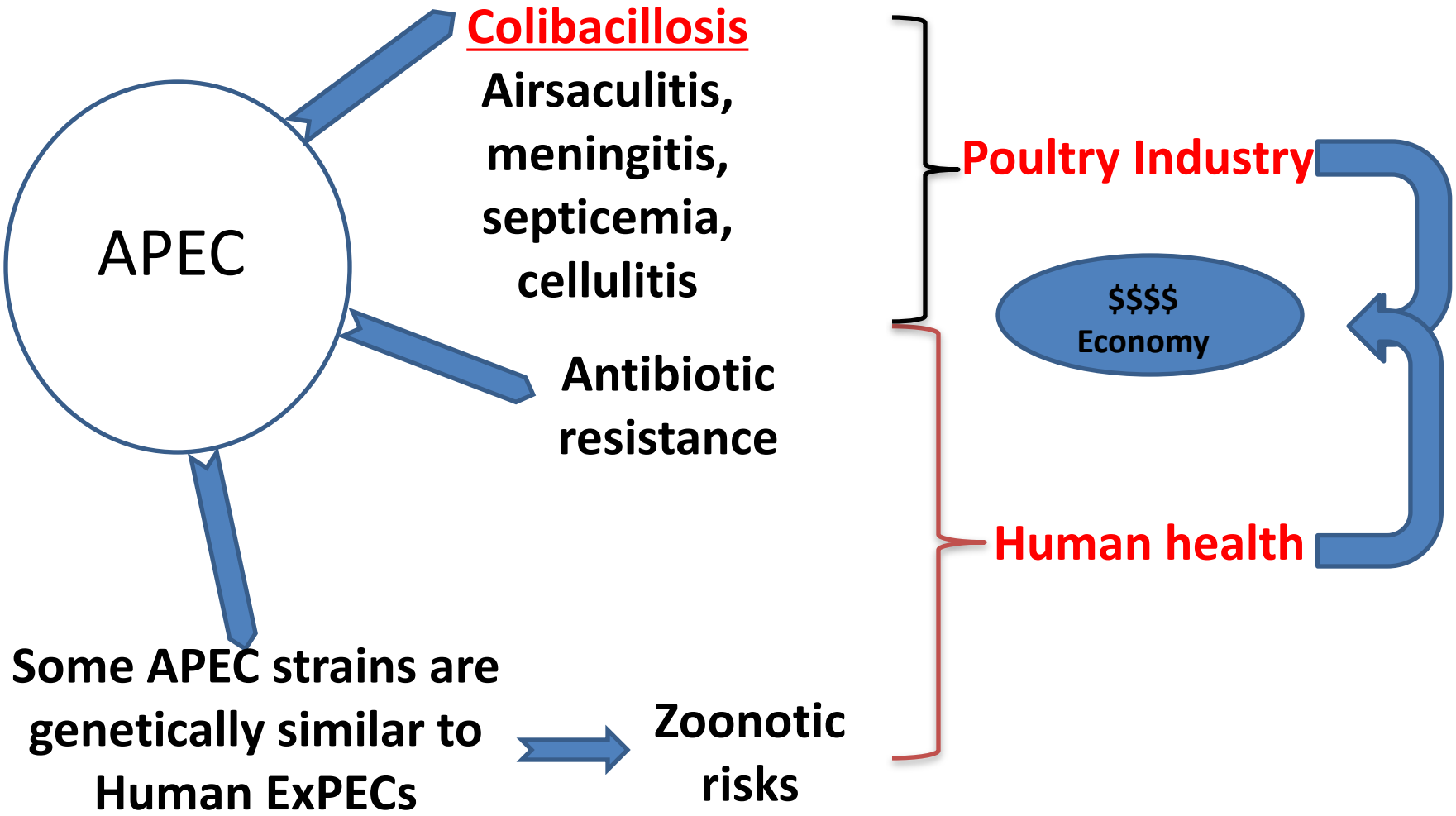
Potential Human Pathogens Transmitted through the Food Chain

- *Salmonella enterica* serotypes
- *Escherichia coli* pathovars
- *Campylobacter jejuni*
- *Clostridium perfringens*
- *Listeria monocytogenes*
- *Enterococcus sp.*

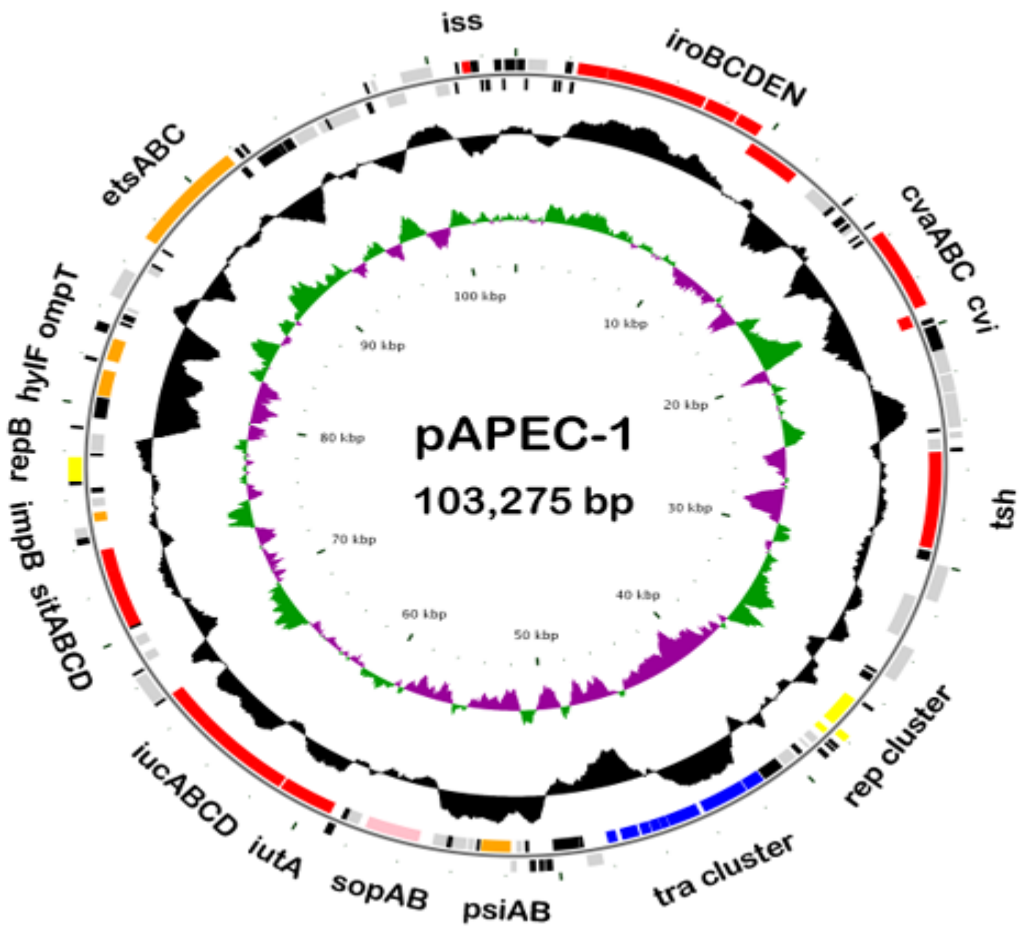
Proper food handling, good personal hygiene, improved sanitary engineering and use of vaccines would probably curtail these infection problems.

Avian Pathogenic E. coli (APEC)

Subgroup of Extra-intestinal pathogenic *E. coli* (ExPEC) that cause diseases in poultry, other animals and humans



pAPEC-1 is a large *E. coli* virulence plasmid present in APEC and ExPEC pathovars



Fur and MntR regulated Iron and Manganese acquisition systems

iroBCDEN (Siderophore, salmochelin)

iucABCD iutA (Aerobactin)

sitABCD (Iron and manganese transport)

Tsh (adhesin/protease/heme binding protein

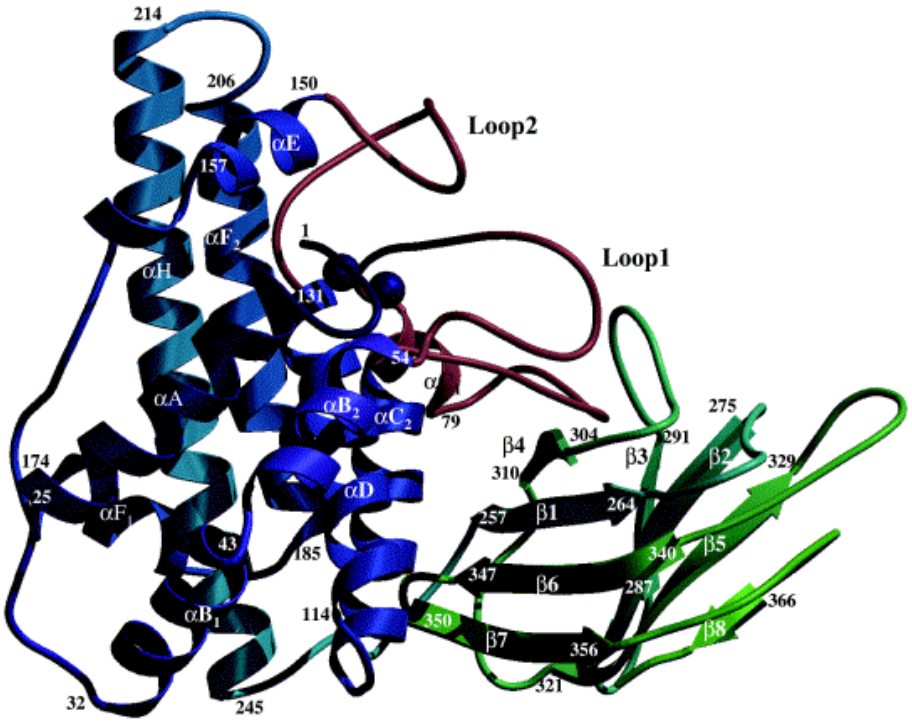
Clostridium perfringens

- Type A *C. perfringens* that produce α-toxin are responsible for enteropathology in animals and humans ranging from sub-clinical intestinal mucosal damage to necrotic enteritis.

- Blockage of the α-toxin C-terminal domain with antibodies, neutralizes both phospholipase and hemolytic activities.

- We constructed an oral RASV delivering the C-terminal fragment of α-toxin that elicits toxin-neutralizing antibodies that suppresses replication of *C. perfringens and* induces protective immunity against necrotic enteritis in chickens. We have improved this vaccine to deliver the NetB protective toxin antigen and the Fba protective antigen identified by John Prescott's lab at Guelph.

Necrotic enteritis toxin

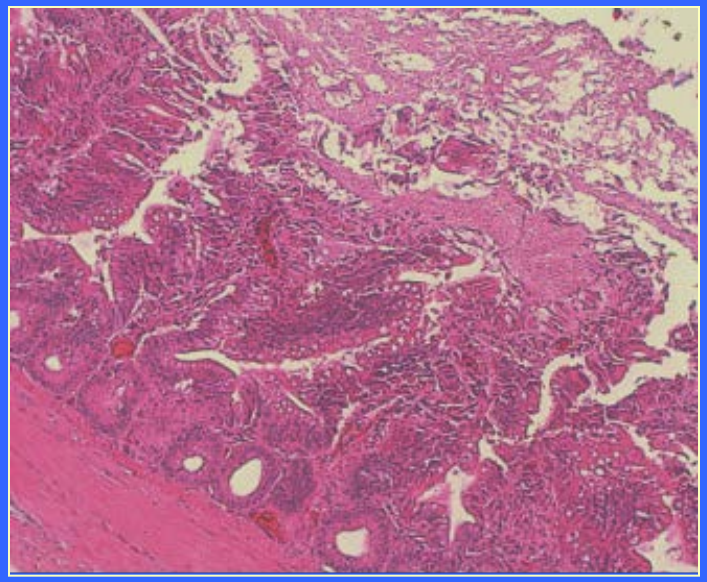


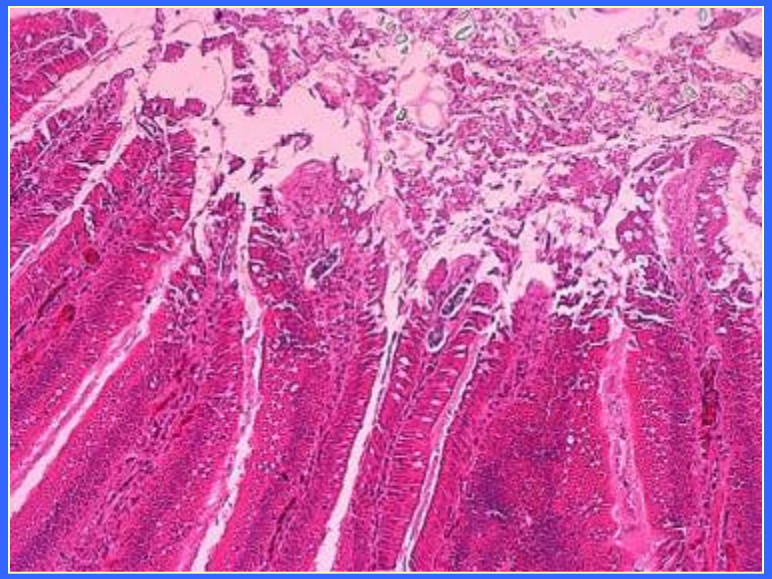
•Figure: Ribbon diagram of the closed structure of alpha-toxin. Blue - N-terminal domain, residues 1–245. Green - C-terminal domain, residues 250–370 (Eaton, JT., et al., 2002. J. Mol. Biol. 319: 275 – 281)

The N-terminal domain by itself has no haemolytic and toxic properties, but still exhibits phopholipase activity. The truncated C-terminal domain has no toxic or enzymatic activities but induces immune protection against necrotic enteritis.

Histopathology

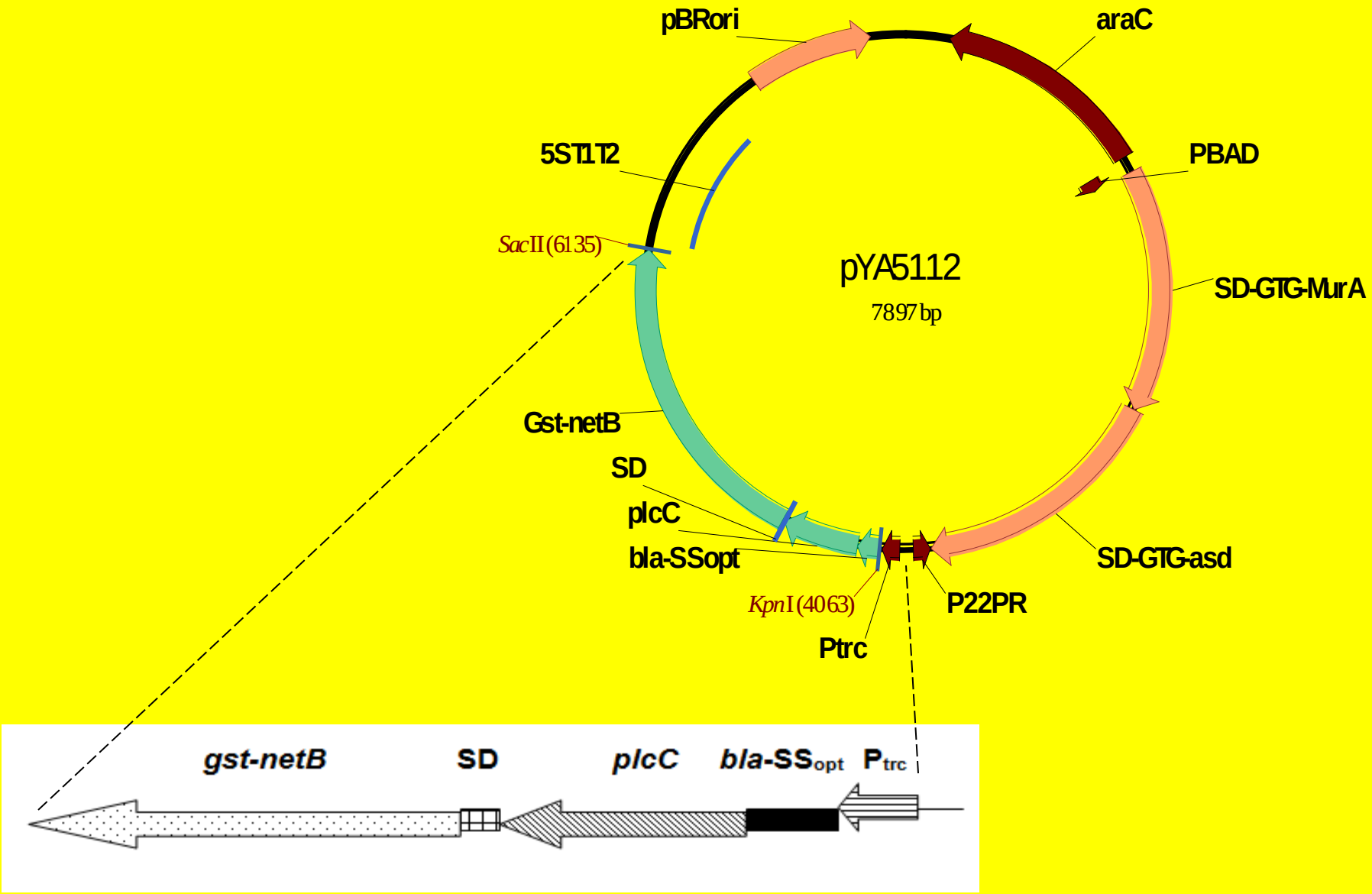
Normal mucosa - Distal jejunum





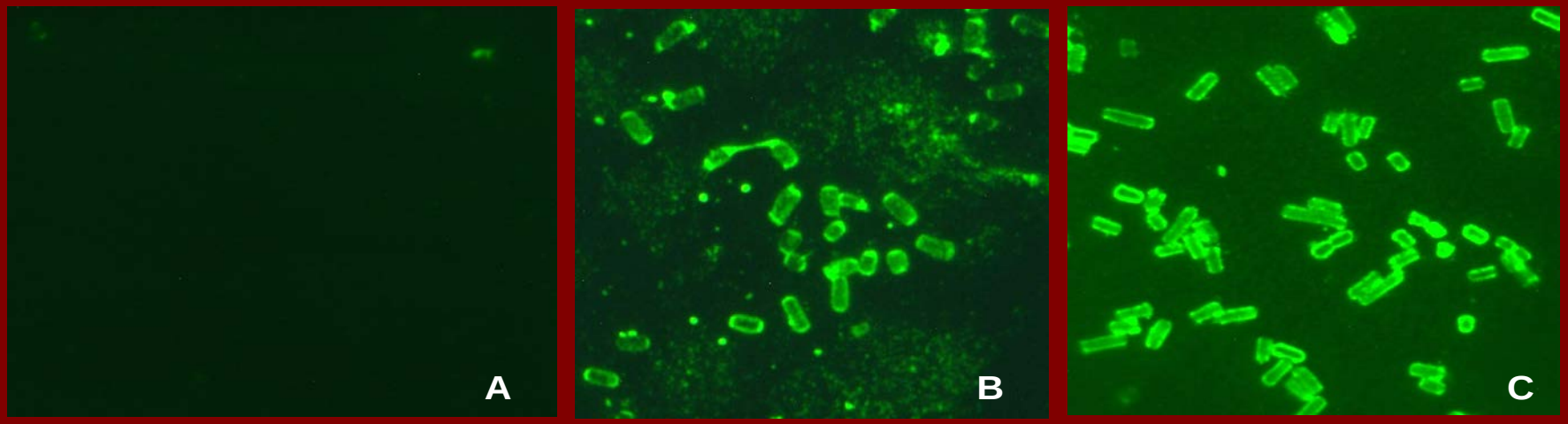
Epithelial degeneration, necrosis, inflammatory leukocytes in lamina propria, enterocyte sloughing, villi fusion, shortening

plcC and *netB* operon fusion for antigen delivery by lysis



Immunofluorescence microscopy

Serum antibody from chickens immunized with rPlcC or RASV bind to bacteria surface



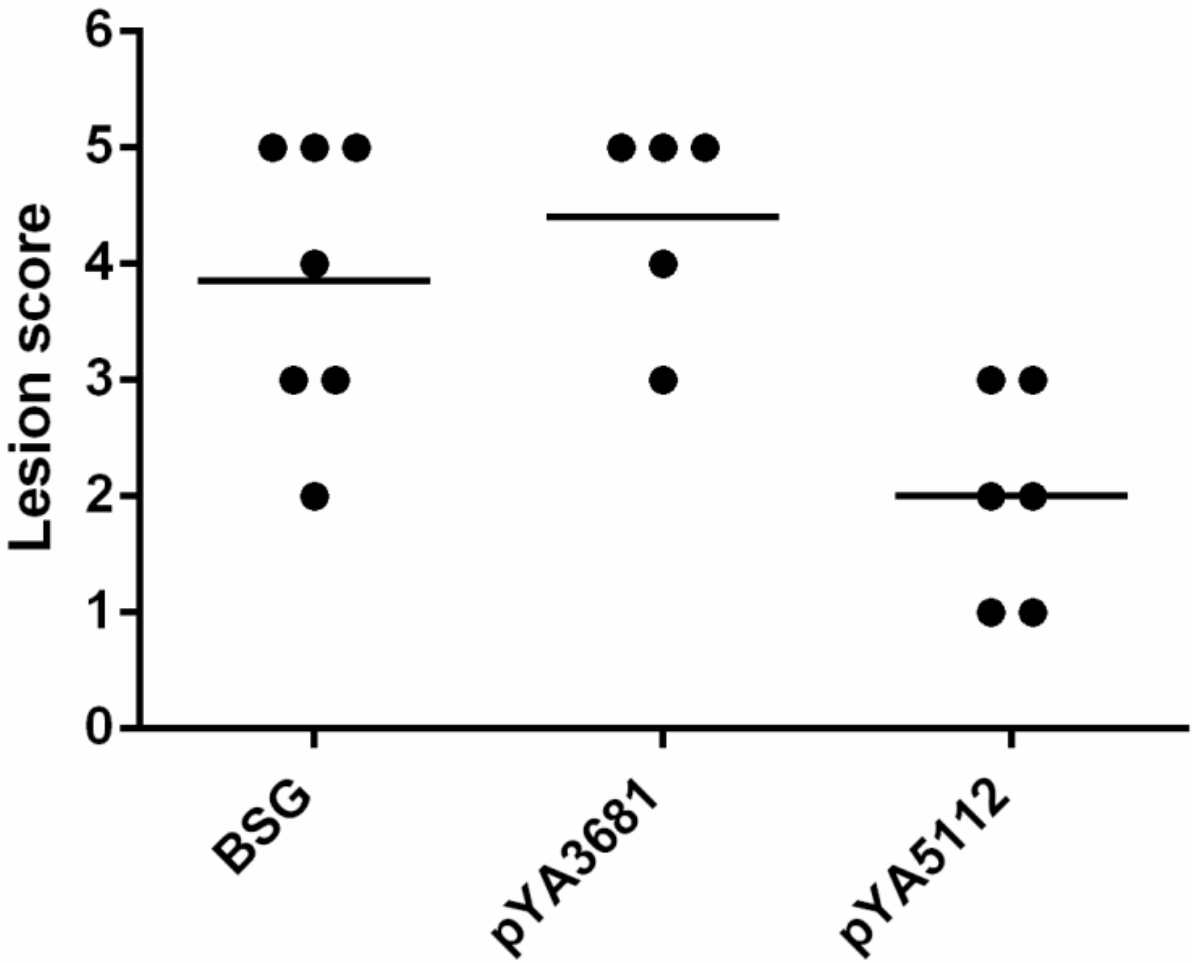
Immunofluorescence showing reaction of serum antibody with *C. perfringens* bacterial cell surface

A. Serum from non-immunized control chicken

B. Serum from rPlcC protein injection immunized chicken

C. Serum from RASV immunized and rPlcC boosted chicken.

Protection against *C. perfringens* challenge in broiler chickens



Broiler chicks were orally immunized at 2 days of age and boosted at 14 days of age. Birds were challenged on days 28-32 with *C. perfringens* strain CP4* and scored on day 33.

BSG: no vaccine control;
pYA3681: empty vector control;
pYA5112: *plcC, netB* operon fusion

Vaccine strain χ11802 :
ΔP_{murA25}::TT *araC* P_{BAD} *murA* Δ*asdA27*::TT *araC* P_{BAD} *c2* Δ(*wza-wcaM*)*-8* Δ*pmi-2426* Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*recF126*

*CP4 was isolated from a field case of necrotic enteritis

Other Food Safety Vaccines

A Recombinant Attenuated *Salmonella* Vaccine (RASV) to reduce the prevalence of *Campylobacter jejuni* in cecal contents of poultry is very much needed. *C. jejuni* increases in titer as a consequence of stress in poultry being crated and trucked to processing plants and fecal contamination of feathers and skin leads to a high incidence of carcass contamination. *C. jejuni* is the major cause of bacterial diarrheal disease in the US. We are thus constructing a RASV-Cj Food Safety vaccine.

Course spray vaccination of day-of-hatch chicks in hatchery

Vaccines Under Development

- **Animal and Human live non-recombinant and RABVs to prevent infection and diseases by:**
  - ***Salmonella enterica*** **Typhimurium, Gallinarum, Choleraesuis, Paratyphi A & Typhi**
  - ***Streptococcus pneumoniae*** **(RASV)**
  - ***Mycobacterium tuberculosis, avium & leprae*** **(RASV)**
  - ***E. coli*** **APEC, ExPEC, UPEC, ETEC, EPEC, EIEC (RASV)**
  - ***Clostridium perfringens*** **(RASV)**
  - **human and avian influenza (RASV)**
  - ***Shigella*** **sp. and serotypes (RASV)**
  - ***Yersinia pestis, enterocolitica*** **and** ***pseudotuberculosis*** **(RASV & RAYV)**
  - ***Campylobacter jejuni*** **(RASV)**
  - ***Taenia solium*** **(human tapeworm) (RASV)**
  - ***Edwardsiella tarda*** **and** ***ictaluri*** **and** ***Flavobacterium columnare***
  - ***Giardia lamblia*** **(RASV)**

- **The RABV for animals will be derived from *S.* Typhimurium UK-1, S. *Gallinarum, S.* Choleraesuis, *Y. pestis* or *pseudotuberculosis* and *E. ictaluri* and for humans from *S.* Typhimurium UK-1 or *S.* Paratyphi A or *S.* Typhi**

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