Hijacking the Type VI Secretion System to develop a Bacterial Anti-Cancer Therapy

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Introduction

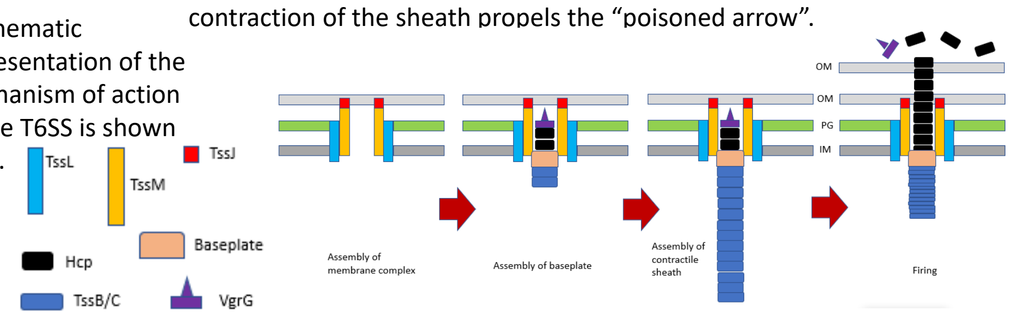
The goal of my project was to engineer a bacterial strain able (i) to colonize the human colon, (ii) to specifically bind to colon cancer cells, and (iii) to deliver a toxin to kill said cancer cells.
For this, I need:

1. a disabled strain of a diarrheal *E. coli* species (lacking the main toxin that causes the disease), able to enter and to live in the colon. I used the 17-2 non-pathogenic enteroaggregative *E. coli* (EAEC) strain.
2. to expose at the cell surface an antibody that specifically recognizes the carcino-embryogenic antigen (CEA5), a marker of colorectal cancer cells.
   - I will use the HbpB autotransporter to display an anti-CEA5 nanobody (nbCEA5) on the surface of E. coli – **this recognizes the colorectal cancer cells**.
3. to deliver an eukaryotic-targeting toxin using the EAEC anti-bacterial Type VI secretion system (T6SS) such that it is effective at killing/disabling eukaryotic cells
   - I will fuse an actin crosslinking domain (ACD) to the VgrG spike of the T6SS

Introduction – Type VI Secretion System

The T6SS is a multiprotein apparatus that functions as a "nano-crossbow" [1]. It is used by bacteria to inject toxins or other effectors into target cells using a contractile mechanism: the contraction of the sheath propels the "poisoned arrow".

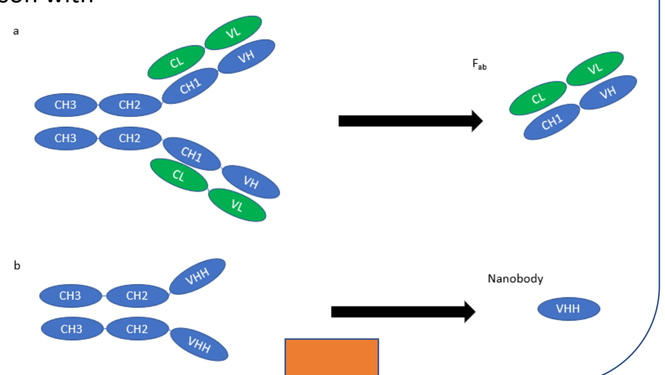
A schematic representation of the mechanism of action of the T6SS is shown here.



Introduction – Nanobody

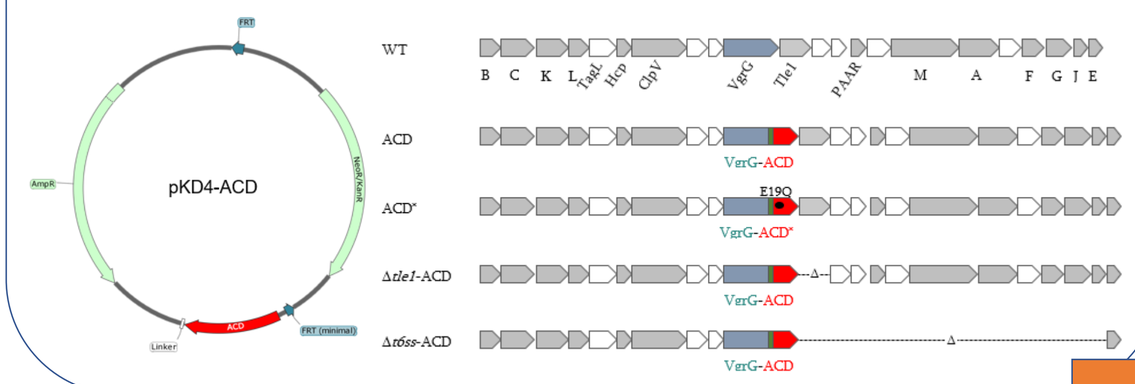
Whereas typical antibodies are usually about 150-kDa in size and composed of multiple chains, nanobodies [2] are composed of a single domain and are roughly 15 kDa in size. Nanobodies are the VHH or variable heavy region of the heavy chain of antibodies produced by camels and similar species, and a basic structural comparison with a "typical" antibody is shown here.

For my project, the use of a nanobody was preferable to any other type of antibody, because the nanobody DNA sequence is short and so relatively easy to insert on the plasmid and chromosome, and also because the simple structure of the antibody makes it likely that *E. coli* will be able to fold and express it correctly.
The CEA5 nanobody being used in my project has already been described [5]



Genetic Constructs

During the course of my research project, I engineered the pKD4-ACD vector that allows chromosomal insertion of the *Vibrio cholerae* ACD toxin-encoding gene. I then constructed 4 new strains of E. coli, using the λ red system encoded on the pKOBEG plasmid (not shown here). These include the VgrG-ACD construct, as well as three controls: a strain with an inactive form of the ACD (mutation of the E19 catalytic residue), and strains with deletion of the tle1 phospholipase or T6SS genes. The 4 constructs that I created are diagrammed below in comparison with the wild type *E. coli* T6SS gene cluster.



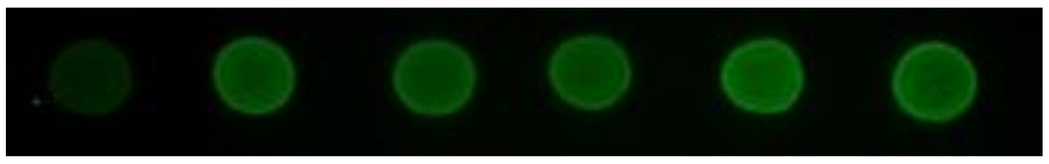
Introduction -Autotransporter

To display the nanobody on the surface of the *E. coli*, I will fuse the nanobody to an **autotransporter**, a bacterial outer membrane protein which has the ability to form a pore in the membrane, and then send a "passenger domain" through the pore for display on the bacterial surface. Thus, the nanobody will be fused to the passenger domain of the autotransporter.
The specific autotransporter I will use is HbpB, which has already been described [6]

Antibacterial Competition assay

I first tested whether the T6SS was functional in the strains that I had engineered. Because the EAEC T6SS has been shown to have anti-bacterial activity (by delivering the anti-bacterial phospholipase Tle1), I performed a fluorescence killing assay using a fluorescent prey strain [3]. The idea is that the fluorescent prey strain is mixed with the predator strain on a spot, and after incubation the fluorescence is measured. The greater the killing activity of the predator strain, the more of the fluorescent prey will killed, and thus the less fluorescence will be observed on the spot.

Predator:	WT	Δ*tssK*	ACD	ACD^{E19Q}	ACD Δ*tle1*	ACD ΔT6SS

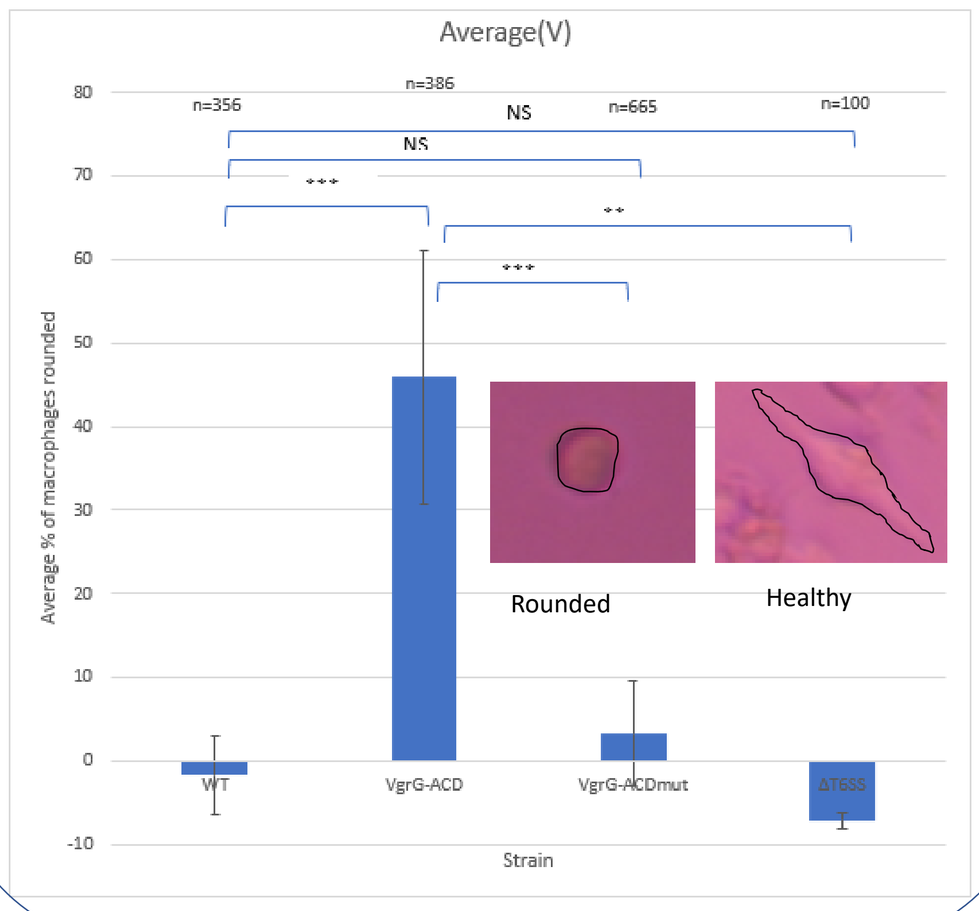


At a first glance it would appear that all the engineered strains are unable to kill competitors, because they have fluorescent levels comparable to the T6SS inactive mutant (Δ*tssK*). However, this could be due to (i) an inactive T6SS, or (ii) the inability of the T6SS to deliver the Tle1 toxin because I have inserted the ACD domain at the normal position of Tle1 on the VgrG spike. Note that ACD-Δ*tle1* not expected to display any killing activity against bacteria.

Another way of testing whether the T6SS is functional would be to test the medium for the presence of secreted T6SS effectors or to visualize sheath dynamics by fluorescence microscopy.

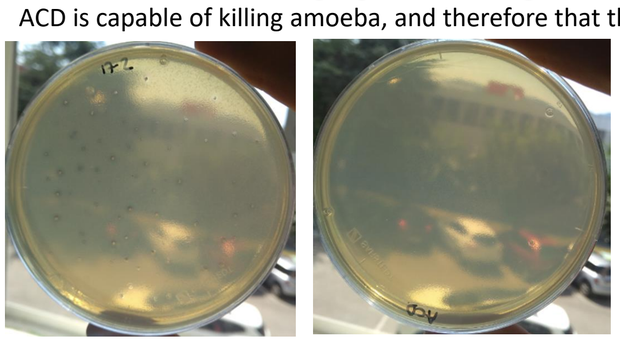
Macrophage Rounding assay

In this assay, the predator strains were incubated with macrophages. The idea here is that if the bacteria have a functional T6SS, then the bacteria should translocate the ACD domain into the macrophages, resulting in actin cross-linking. Since macrophages use actin to maintain their shape, this would result in an observable phenotype where the macrophages lose their "rough" appearance and become rounded [4].
The results shown below demonstrate that only the wild-type strain with the ACD insert causes macrophage rounding. The observation that the ΔT6SS and ACDE19Q strains do not cause macrophage rounding further highlights that rounding requires both an active T6SS and functional ACD toxin. Thus, I conclude that the T6SS delivers the ACD domain inside macrophages, and that this causes actin crosslinking in these macrophages.



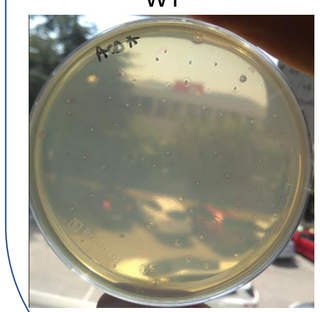
Amoeba Predation assay

After the promising results of the macrophage rounding assay, I further investigated the anti-eukaryotic activity of the VgrG-ACD strain by testing its ability to resist predation by amoeba. I first created a lawn of bacteria on the agar plate, then spread a solution of amoeba over the top of this, and incubated overnight. In the WT condition, the amoeba predate the bacteria and thus plaques appear in the bacterial lawn. However, if the ACD strain has anti-eukaryotic activity, then it should be able to kill the amoeba and thus there will be no or few plaques in the bacterial lawn after overnight growth. Here again, I showed that only the strain with an active T6SS and an active ACD is capable of killing amoeba, and therefore that the ACD domain is translocated inside amoeba.

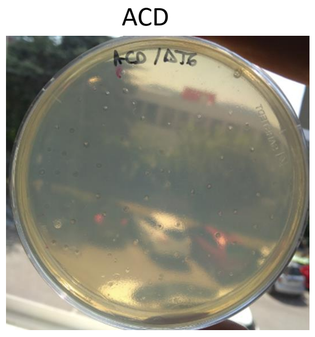


WT

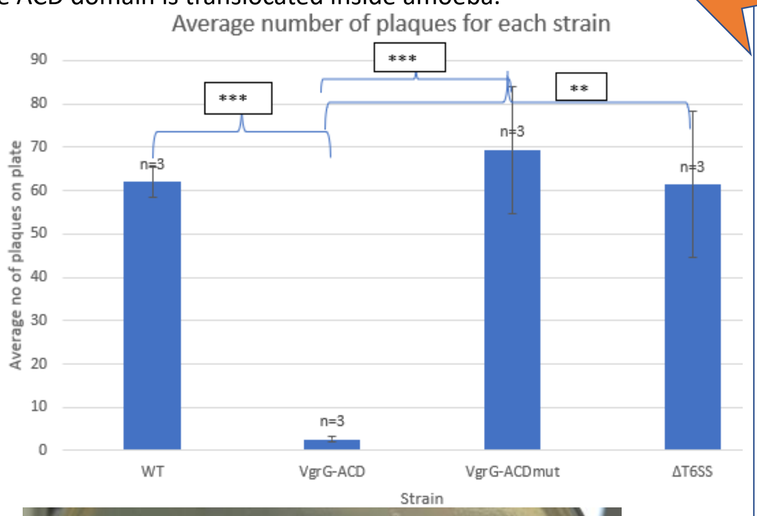
ACD

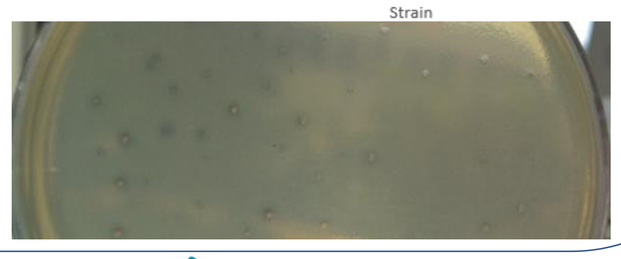


ACD^{E19Q}



ACD - ΔT6SS





What next?

VgrG-ACD construct

- Test constructed strains for actin labelling in fibroblasts
- Test strains for virulence towards *C. elegans* and mice.

Autotransporter-nanobody construct

- Insert nanobody into pKD4-Sci1 Autotransporter plasmid
- Continue to work with construct

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THE CAREERS SERVICE

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