The Importance of YscD in the Type III Secretion System

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Background

• A Type III Secretion System (T3SS) is a system within a bacteria that allows it to inject host cells with proteins. These proteins then 'disable' the immune system, leaving the host cells susceptible to disease. T3SS encodes a specific system, made up of a base which spans the bacterial inner and outer membranes, and a needle which extends outside of the bacterial surface, this is often referred to as an injectisome. Once activated the T3SS begins to form injectisomes and secrete proteins.

• Yersinia is a bacteria that is related to many diseases such as the plague, which is caused by Y. pestis, as well as stomach and intestinal pains caused by Y. enterocolitica and Y. pseudotuberculosis.

• YscD is a protein that is found within Yersinia's T3SS. YscD is embedded within the inner membrane of the bacteria.

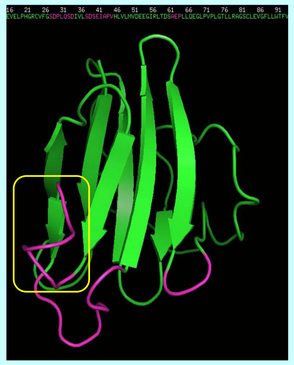
• YscD is an important part of Yersinia's T3SS, because when YscD is removed from Yersinia, it is no longer able to secrete proteins.

• The structure of the YscD protein resembles that of a Forkhead Associated Domain (FHA Domain).

• It has been found that FHA Domains tend to bind with other proteins at loops 3, 4, and 5 in the FHA Domain. The proteins that bind are usually phosphorylated threonines or the FHA domain can be phosphorylated themselves.

Objective

• Test the importance of the third loop in the YscD protein.



Introductory

After learning that the protein YscD is important, it will be beneficial to learn which amino acids in the protein are vital to bacterial infection. This can be tested by replacing different amino acids with alanines. Alanines are chosen to replace, certain amino acids in YscD because alanines cannot bind with the other proteins. Because it has been found the Forkhead Associated Domains bind at their loops 3, 4, and 5 those are the parts of the protein that will be tested first. Loop 3 is the shortest of the three loops with seven amino acids and will be the focus of this work.

Procedure

- Introduce mutations in gene encoding the third loop using PCR and primers encoding alanines. The YscD gene is going to be amplified with the alanine mutations from the plasmid pBAD which includes the wild type YscD gene.

1. Create a Reaction Mixture:
   - -5 mL of 10x Buffer
   - -1 mL of Plasmid Template (DNA)
   - -0.8 mL of Primer Forward
   - -0.8 mL of Primer Reverse
   - -4 mL of Water
   - -1 mL of PFU (enzyme)

Original: TTT GGT TCA GAC CCG TTG CAG TCA GAT ATT GTT

Mutated: TTT GGT GCA GCA GCG GCG GCG GCA GCT ATT GTT

2. Use a Polymerase Chain Reaction Machine (PCR Machine)

Segment	Cycle	Temperature	Time
1	1	95 C	2 minutes
2	30	95 C 58 C 68 C	1 minute 1 minute 8.5 minutes
3	1	68 C	5 minutes

S D P L Q S D becomes A A A A A A A

3. Remove from PCR and add 2 uL of DPN1
4. Leave in a 37 C bath for approximately 2 hours.
5. After 2 hours, remove from the warm bath and add 4 mL of this reaction to E. coli competent cells. Allow the solution to sit in ice for about 20 minutes.
6. After 20 minutes, heat shock E. coli at 42 C and return to ice.
7. Add 1 mL of LB Broth and shake at 37 C for 45 minutes
8. After 45 minutes pour the solution onto an ampicillin containing LB plate. Be sure that the environment near where the solution is poured is sterile.
9. Store overnight at 37 C and check to see if colonies of bacteria form on the plates.
10. If colonies of bacteria form, then pick the colonies and grow in LB with 50 ug/ml ampicillin overnight.
11. After the E.coli has grown, centrifuge and retain the bacterial pellet. Use a mini-prep kit to isolate the DNA .
12. Send the plasmid DNA for sequencing. Check the sequence to see the if the gene encoding the third loop amino acids have mutated into alanine encoded base pairs.
13. Add the mutated YscD-pBAD plasmid to YscD-Knock Out strain of Yersinia via transformation.
14. If Yersinia can still secrete proteins, it means that loop 3 is not significant. However, if no secretion occurs after the mutated YscD is placed in Yersinia, then it proves that loop three is vital to Yersinia's T3SS.

Trial 1

In the first trial, the procedure was followed exactly as written; however, no colonies grew after step 10.

Trial 2

For the second trial three major changes were made. The amount of plasmid template added in step one was decrease to 0.5 uL and the amount of DTNP's was increased to 4 uL. Also, the amount of time for the first two parts in segment two were decreased from one minute to 45 seconds. After transformation, 8 colonies were picked and grown in LB. The bacteria was harvested and the plasmid purified by a mini-prep kit. The DNA was sent to Genewiz for sequencing and unfortunately, none of the results showed any mutation of the third loop.

Trial 3

The changes made in trial 1 were kept the same. After the transformation, one colony was picked, grown, mini-prepped, and sent in for sequencing. Once again, the results showed no change in the third loop.

Trial 4

In the fourth trial there were 4 varying samples instead of having all of the mutagenesis reaction the same. The variations can be find in the amounts of plasmid and primer added to the PCR reaction.

Sample 1
-0.5 mL of 90ng/mL
-0.5 of each pair

Sample 2
-0.5 mL of 45 ng/mL
-0.5 mL of each pair

Sample 3
-0.5 mL of 90 ng/mL
-1 mL of each primer

Sample 4
-0.5 mL of 45 ng/mL
-1 mL of each primer

Also, the PTC-100 PCR was used instead of the TC-3000, which is the PCR used in previous trials. This changes was made do to the possibility that the TC-3000 may be faulty. All of the other steps will be kept the same.

After the transformations, no colonies were formed.

Related Work

Apart from the main experiment, other related experiments were conducted in order to discover different aspects of the YscD protein. An attempt was made to purify the protein, however, this is a lengthy process that took up much of the down time left after the main experiment. First the bacteria must be grown. Then the bacteria was centrifuged down for ten minutes in order to remove media and collect the bacteria from the pellet. Afterwards buffer was added to re-suspend the pellet. This solution was lysed using the emulsiflex. Then the lysed cells were centrifuged to get rid of the inclusion bodies. At this point the purification can go in two different directions. The protein YscD is mostly located in the membrane, so the supernatant was ultra-centrifuged so that all of the pellet will have the membrane. Then membrane is resuspended and put through an anion exchange column. The column will allow the proteins to come out with different concentrations of salt. Samples from each salt concentration were collected and run on a gel. It was found that the protein YscD usually comes out around 160 mM NaCl; however the gel was too indefinite, so it was decided that a Western Blot should be run. Western Blots go through a similar process, however after running a gel, the gel is not stained, and is instead put on a membrane. The membrane is soaked for varied amounts of time in primary antibodies and secondary antibodies before it is developed. The first attempt at a Western Blot was not successful, because the before the Western Blot was developed, the antibodies used were not strong enough. The second attempt at a Western was unsuccessful as well, except in the case of the second attempt, the antibodies were too concentrated. Another attempt at a Western Blot is ongoing right now, with hopes that in this attempt, the Western will develop nicely and the location of the YscD protein band will finally be visible

Conclusions

YscD is important in Yersinia's T3SS. However, from the evidence collected from the experiments, the importance of loop 3 in the YscD protein is not as apparent. There is evidence from literature that it may be important, but due to the limited data collected from the four trials, there is no concrete evidence that loop three, in particular, is important. Since YscD resembles a FHA structure, there is a large probability that either loops 3, 4, or 5 are important.

Future Research

In the future, it is possible that the importance of loop three will be found. If loop 3 is important, then it implicates that it is involved in reactions with other T3SS. However, if loop three is found to have little or no importance, then loops 4 and 5 will go through similar testing until one of the loops prove to be vital to Yersinia's T3SS.