Lipopolysaccharide binding site identification and Colicin N Toxicity

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Introduction

- Outer Membrane Protein F (OmpF) is a trimeric protein found in the outer membrane of *Escherichia coli,* a gram negative bacterium (1)
- Colicin N is a 42kDa antibacterial protein (2).
- Colicin N initially binds to OmpF bound lipopolysaccharide (LPS) in the outer membrane (See Figure 7)(2), which can result in cell death through the formation of a voltage dependent channel in the outer membrane (3)
- Identification of the LPS binding site and therefore where Colicin N binds may allow for future antibiotic development.

Aim

- Identify by using SDS page whether the mutants shown in yellow and purple (shown in figure 3) bind Lipopolysaccharide (LPS)
- Identify whether Colicin N is toxic to the mutants created

Cell	Mutant	Mutation
BZB1107	Alanine Complete	Positive to a non polar, neutral charge
BZB1107	Glutamate Complete	Positive to negative charge
BZB1107	Alanine A	Positive to a non polar, neutral charge at site A
BZB1107	Glutamate A	Positive to negative charge at site A
BZB1107	Alanine B	Positive to a non polar, neutral charge at site B
BZB1107	Glutamate B	Positive to negative charge at site B
BZB1107	Glutamine A	Positive to a polar, neutral charge

Figure 1: Table showing the mutants used
Note, the Glutamine A mutant was researched before my project commenced, but is included here for comparison

Method

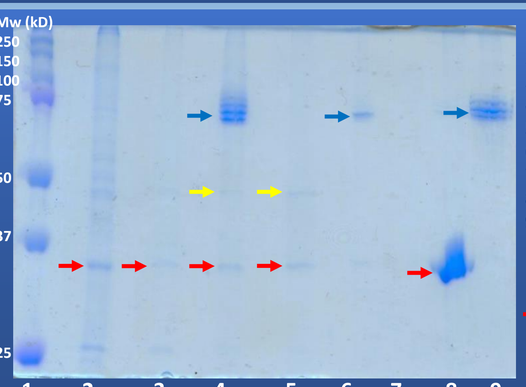
SDS page- Lipopolysaccharide Binding

1. Plasmids carrying the OmpF mutant genes were inserted by transformation into BZB1107 *Escherichia coli* cells and allowed to grow once induced with IPTG
2. Purified by homogenization of the membrane fraction and OmpF extract collected
3. 10µl of extract run on a 10% SDS polyacrylamide gel
4. The remaining extract was concentrated by ethanol precipitation and 10µl run on a 10% polyacrylamide gel

Binding can be identified by the presence of a series of bands which form a ladder. (See Figure 2)

Spot tests- Colicin N toxicity

1. Each transformed mutant along with a positive (Wild type OmpF) and negative (BZB1107 *E.coli)* control was grown up in 5ml LB growth medium with 5µl Ampicillin. The negative control contained no ampicillin.
2. 30µl of each mutant was added to 2ml of LB medium and 2ml of sloppy agar, poured on to individual plates, negative control contained no Ampicillin
3. Each plate was then 'spotted' with 2µl of varying amounts of Colicin N: 5000ng, 500ng, 50ng and 5ng with phosphate buffer acting as a control on each plate
4. The plates were then incubated overnight at 37°C, to allow for killing (See Figure 4)



1.Marker
2.Alanine Complete- Unboiled
3.Glutamate Complete- Unboiled
4.Alanine A- Unboiled
5.Alanine B- Unboiled
6.Glutamate A- Unboiled
7.Glutamate B- Unboiled
8.OmpF Control- Boiled
9.OmpF Control- Unboiled

Figure 2: SDS gel showing all mutants run at a concentration of 2mg/ml
→=Monomer →=Dimer →= Trimer
(Note, for lane 7 the correct concentration could not be reached, resulting in a faint or absent line.)

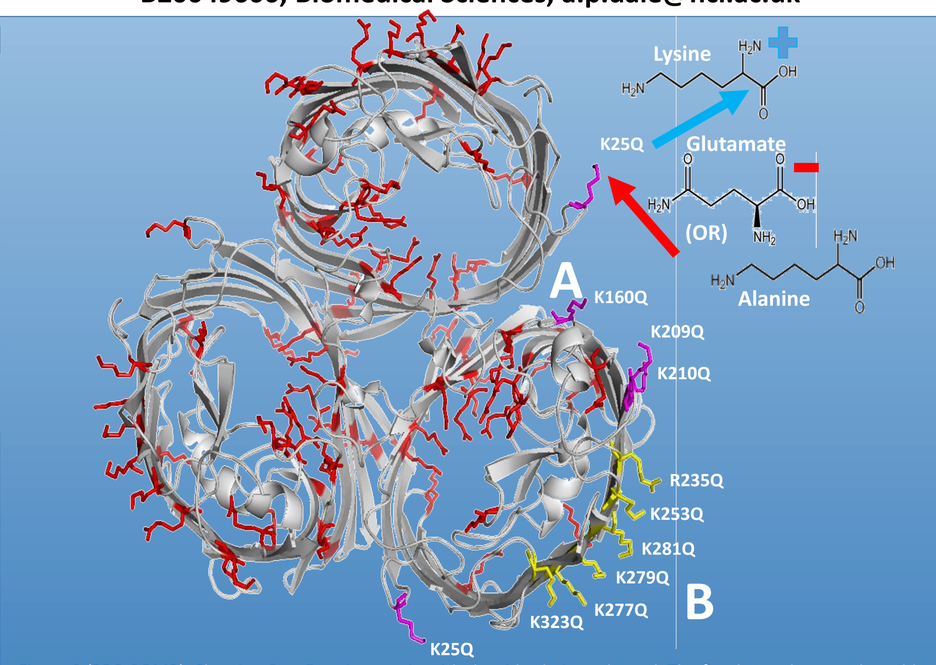
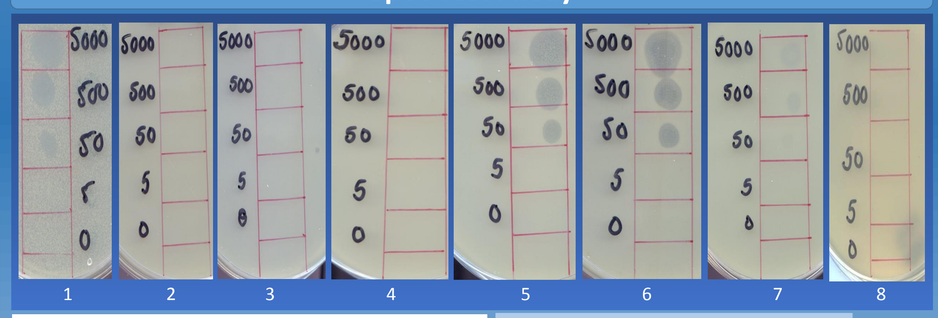


Figure 3 (PDB:2OMP): Showing OmpF porin protein with the side chains coloured. The first letter denotes the wild type residue and the number denotes the location of the residue in the sequence. Site A and B denote the location of mutations. Site A is K25Q, K16Q, K209Q, K210Q. Site B is R235Q, K235Q, K281Q, K279Q, K227Q, K323Q
Mutation shown at the top of the diagram is repeated at each mutation site respectively.

Spot Test Assay



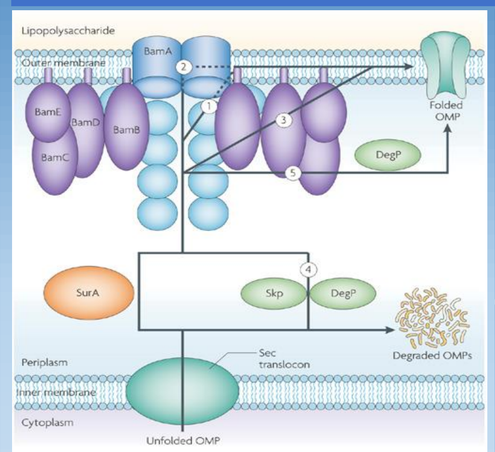


Figure 4: Spot Test Assay: Each spot indicates killing by Colicin N. 5000-0 indicate the amount of Colicin N 'spotted' in ng.
1. Negative control (BZB1107 *E.coli* cells)
2. Positive control (Wild Type OmpF)
3. Alanine Complete
4. Glutamate complete
5. Alanine A
6. Glutamate A
7. Alanine B
8. Glutamate B

Figure 8: Current proposed transport pathway of unfolded OMP through the periplasm to form folded OMP in the outer membrane (Timothy J. Knowles, A. S.-T., Michael Overduin & Ian R. Henderson (2009). "Membrane protein architects: the role of the BAM complex in outer membrane protein assembly." Nature Reviews Microbiology **7**: 206-214)

Results

SDS PAGE

Mutant	LPS Binding	Structure
Glutamate Complete	No	Monomer
Alanine Complete	Yes	Monomer, some Trimer
Alanine A	Yes	Monomer, Dimer, Trimer
Alanine B	Yes	Monomer
Glutamate A	Yes, less than normal	Monomer, Dimer, Trimer
Glutamate B	Yes	Monomer
Glutamine A	Yes, less than normal	Trimer

Figure 5: Table showing results of SDS Page
(Note, the Glutamine A mutant was researched before my project commenced, but is included here for comparison)

Spot test Assay

Mutant	Killing	Minimum amount of Colicin N which killed (ng)
Glutamate	No	n/a
Alanine	No	n/a
Alanine A	Yes	50
Glutamate A	Yes	50
Alanine B	Yes	500
Glutamate B	No	n/a

Figure 6: Table showing results for Spot Test Assay

Conclusion

SDS page

- Mutation of the side chains at site A and B effects LPS binding and can influence overall structure formation
- The LPS binding site is present at site B
- Mutations at site B prevents trimer formation, therefore only monomers can be formed.

Spot test

- Killing by Colicin N can only take place when LPS is bound to OmpF, and only when it has been successful folded into a trimer

Further Work

- Carry out further mutations of specific side chains in site B, in order to help identify the specific binding site of LPS
- Identify whether the monomers formed are folded or unfolded
- Research further how the transport pathway of unfolded Outer Membrane Protein (OMP) through the periplasm to form folded OMP in the outer membrane can be disrupted, leading to misfolding or degradation. (See figure 8: the current proposed transport pathway)

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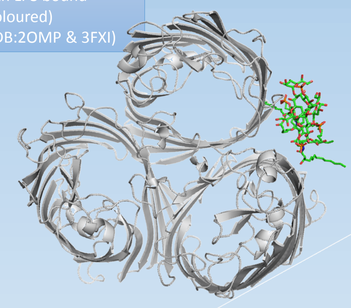
Figure 7: OmpF (grey) with LPS bound (Coloured) (PDB:2OMP & 3FXI)



References
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(2) Baboolal TG, Conroy MJ, Gill K, *et. al.* Colicin N Binds to the Periphery of its receptor and translocator, Outer Membrane Protein F. *Cell Press: Structure* 2008;16: 371-379
(3) (4) Eric Cascales, S. K. B., Denis Duché, Colin Kleanthous, Roland Lloubès, Kathleen Postle, Margaret Riley Stephen Slatin,Danièle Cavard1 (2007). "Colicin Biology." Microbiology and Molecular Biology Reviews **71**(1)