Molecular analysis of surface proteins of *Clostridium difficile*

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Introduction

Clostridium difficile , a gram positive spore-forming anaerobic bacterium, is the most common pathogen identified as the cause of antibiotic-associated diarrhoea, pseudomembranous colitis and hospital-acquired diarrhoea. The best characterised virulence factors are the large cytotoxins A and B that trigger intestinal inflammation and fluid loss. However, variation in the sequence and predicted biological activity of these proteins is evident from molecular characterisation of isolates *C. difficile* and some outbreak strains carry deletions in one or both of the toxin structural genes. It is likely that other bacterial factors are involved in the disease process, most likely those proteins that are located at or associated with the bacterial surface since this forms the interface between the pathogen and the host. The goal of this study is to analyse surface proteins of *C. difficile* and investigate their role in the infection process (1-3).

Materials and methods

gDNA of *C. difficile* strain 630 was purified from overnight cultures.

PCR was performed to generate fragments from genes of interest using Promega Phusion DNA polymerase, 2mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate and 1 µM of each primer. Reactions were carried out for 35 cycles consisting of denaturation at 94°C (30 sec), annealing at 55°C (30 sec) and extension at 72°C (1 min) in a ThermoHybrid PX2 thermocycler. Sense and antisense primers carried sequences at their 5' termini for annealing to LIC vector as follows:

Sense primer: 5' GAC GAC GAC AAG ATX – gene specific sequence 3'

Antisense primer: 5' GAG GAG AAG CCC GGT – gene specific sequence 3'

Cloning of targets

For cloning of *C.difficile* genes, pET-32 EK/LIC vector from Novagen was used. The amplified products were purified with QIAquick PCR purification kit (QIAGEN). To prepare the purified PCR products, all were treated with T4 DNA polymerase to generate vector-compatible overhangs on the inserts. After annealing the treated inserts with vector DNA, Nova Blue competent cells were used for transformation. Selection of transformants was carried out by plating on 2XYT agar containing ampicillin. Colony PCR was used to screen candidate clones.

Protein expression and purification

Plasmid DNA from positive clones were purified for transformation into an expression host and sequence analysis. Isolated recombinant plasmids were transformed into BL21(DE3) competent cells and plated onto 2XYT-ampicillin agar. Liquid cultures were induced with 1mM IPTG at an OD of 0.8 for overnight expression at 30°C. Supernatants from sonicated overnight cultures were analysed by Western blotting using anti S-Tag antibody. Prepacked Ni sepharose columns (GE Healthcare) were used to purify target proteins. Columns were washed with 50 mM imidazole and target proteins were eluted with 100-300 mM imidazole. Purified proteins were analysed by SDS-PAGE, Western blotting and mass spectrometry.

Isolation of recombinant antibodies

Tomlinson I and J libraries was used to select antibodies against purified target proteins. Briefly, selection included growing the libraries, selection on immunotubes, three round of selection, screening phage particles by ELISA, and production of soluble antibodies. *E.coli* TG1 was used for the production of phage. To produce soluble antibodies from the selected phage, HB2151 cells were used. Infected HB2151 cultures were induced with IPTG at 30°C for overnight culture. Supernatants from overnight cultures were analysed by dot blot using anti c-myc antibody. To assess the ability of selected antibodies to target proteins, anti c-myc was used in Western blotting. DNA plasmids of the clones were sequenced with LMB3 and gIII primers.

Results

In this project, surface proteins of *C.difficile* thought to have a role in pathogenesis were chosen for expression and as targets for isolation of recombinant antibodies. PCR using Phusion buffer HF generated products of the correct size except for *cwp84* and *fbpA* genes (Fig.1). This problem was solved by a change to GC buffer. For *cwp66*, there was no difference between Taq and Phusion (Fig.2)

PCR reaction using buffer HF

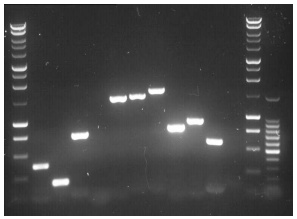


Figure 1: PCR products obtained with sense and antisense primers. Lane 1, 1kb DNA ladder (Promega); lane 2 , *cwp84*; lane 3, *cspA*; lane 4, 5'*cwp66*; lane 5, *fbpA*; lane 6, *groEL*; lane 7, *fliD*; lane 8, *acd*; lane 9, *fliC*; lane 10, 3'*cwp66*; lane 11, *sortaseB*; lane 12, 1kb DNA ladder (Promega); lane 13, 100bp DNA ladder (Promega).

PCR reaction, comparing HF and GC buffer

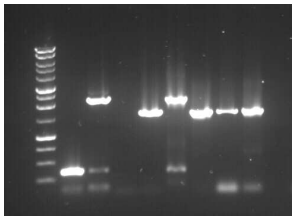


Figure 2: PCR product comparison using HF and GC buffers. Lane 1, 1kb DNA ladder; lane 2, *cwp84* amplified with buffer HF and Phusion enzyme; lane 3, *cwp84* amplified with GC buffer and Phusion enzyme; lane 4, *fbpA* amplified with HF buffer and Phusion enzyme; lane 5, *fbpA* amplified with buffer GC and Phusion enzyme; lane 6, *cwp84* amplified with Taq polymerase; lane 7, *fbpA* amplified with Taq polymerase; lane 8, cwp66 amplified with Buffer GC and Phusion enzyme; lane 9, *cwp66* amplified with Taq polymerase.

The presence of inserts in plasmid DNA from transformed NovaBlue cells was confirmed by colony PCR. This showed the expected molecular weight size of target sequences (Fig. 3). Sequencing confirmed that inserts carried the correct sequence (data not shown).

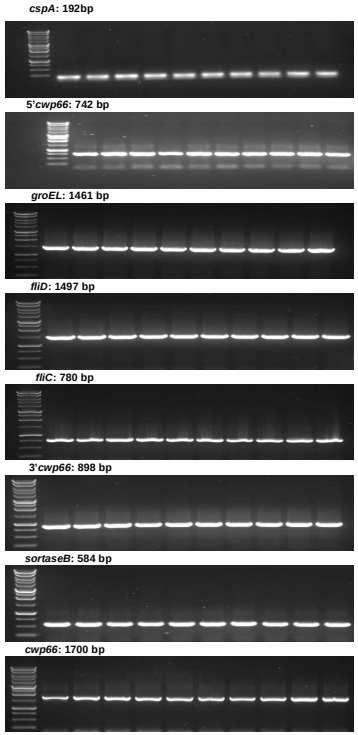


Figure 3. Colony PCR recombinant plasmids. Left lane of each image shows 1kb DNA marker (Promega). Insert sequence is shown in the label for each panel.

SDS-PAGE analysis of recombinant low molecular weight (LMW) and native SlpA

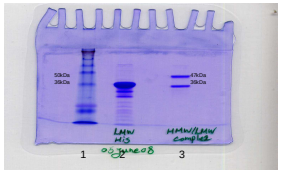


Figure 4. Lane 1, protein marker; lane 2, recombinant LMW (from Prof. N. Fairweather, Imperial College, London); lane 3, SlpA extracted from *C. difficile* cells

Recombinant proteins were eluted from the Ni chelation columns using different concentrations of imidazole and and analyzed by SDS-PAGE (Fig. 5). Purified proteins and LMW SlpA (Fig. 4) were used for isolation of scFvs from phage display libraries. Phage collected from each round of selection were tested by polyclonal phage ELISA for specificity towards the target protein (Fig. 6). Soluble scFv antibodies were expressed in *E. coli* HB2151 and tested in ELISA against the selecting protein target (Fig.7) to assess specific recognition. Based on these results, Western blots were run using scFvs to detect the target protein. In Figure 8, scFvs LG1, LF10 and LG7 were all selected against recombinant LMW, but blotting showed the ability of LG1 to bind the high molecular weight form of SlpA. The CDRs of these antibodies are shown (Fig. 9).

In all, 30 specific scFvs were selected against 8 candidate proteins predicted to be present at the surface of *C. difficile* 630.

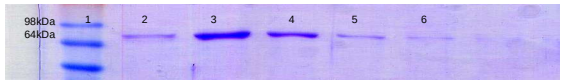
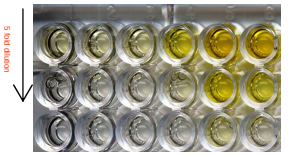


Figure 5. lane 1, pre-stained protein markers (key markers indicated); lanes 2-6, elution of recombinant FliD with different imidazole concentrations.



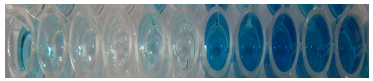


Figure 6. Polyclonal ELISA with phage recovered from each round of selection against SlpA. Library I, columns 1-3. Library J, columns 4-6. 1st round of selection columns 1 and 4; second round of selection columns 2 and 5; third round of selection columns 3 and 6.

Figure 7. Reaction of soluble scFvs against purified *Cwp66*. Wells were coated with Cwp66 and after adding a scFv sample to each well, wells were probed with anti-c-myc, anti rabbit-HRP and TMB substrate to detect scFv binding.

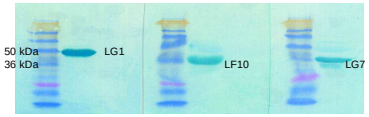


Figure 8. Western blot analysis of three scFvs isolated against recombinant LMW. After SDS-PAGE of SlpA extracted from *C. difficile* cells (Fig. 4, lane 3) and transfer, the membrane was transferred was probed with soluble scFv antibodies LG1, LF10 and LG7 as indicated in each panel. scFv binding was detected with anti-c-myc, anti rabbit-HRP and TMB substrate. Pre-stained markers are in the left lane of each panel, key molecular weights shown.

		----CDR1----	----CDR2----	----CDR3----
LG1	H CHAIN	GFTFSSYA....	IGTYGSST..	AKNAAAFDY
LG1	L CHAIN	QSISSY.....	AAS.....	QQYAYYPTT
LF10	H CHAIN	GFTFSSYA....	IYAGTRT..	AKHPLIFDY
LF10	L CHAIN	QSISSY.....	MAS.....	QQKKATPAT
LG7	H CHAIN	GFTFSSYA....	ISTHGSRT..	AKNGTLFDY
LG7	L CHAIN	QSISSY.....	QAS.....	QQAQSNPQT

Figure 9. Predicted protein sequences of anti-SlpA scFv antibodies. Sequences of CDRs are shown for each scFv analysed in Fig. 8 through heavy (H; green) and light (L; blue) chain domains.

Conclusion

Surface proteins have pivotal roles in the pathogenesis of bacterial infection. We have generated a panel of scFv antibodies against proteins predicted to be present at the surface of *C. difficile*. These reagents will be used to test for the ability to inhibit a range of bacterial properties (*eg* motility) and assessed for strain specificity.

Acknowledgement

We thank the Iranian Ministry of Health and also the Razi Vaccine Research Institute for financial support.

References

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