

1 **Identification of the receptor-binding protein of *Clostridium difficile* phage CDHS-1**  
2 **reveals a new class of receptor-binding domains.**

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10 **Introductory Paragraph:**

11 Phage-bacterial recognition is species-specific, determined by interactions between phage  
12 receptor-binding proteins (RBPs) and corresponding bacterial receptors. RBPs are diverse  
13 and we present data demonstrating the identification and characterisation of a novel *C.*  
14 *difficile* phage RBP. Putative RBP were identified for CDHS-1 and overexpressed, purified,  
15 and polyclonal antibodies were raised and used in phage neutralization assays. Anti-gp22  
16 neutralised CDHS-1, indicating it is the RBP. Immunogold-labelling and transmission  
17 electron microscopy confirmed this enabling visualization of the protein locations. A detailed  
18 structural understanding was obtained from determining the three-dimensional structure of  
19 gp22 by X-ray crystallography. gp22 is a new RBP class consisting of an N-terminal L-  
20 shaped  $\alpha$ -helical superhelix domain and a C-terminal Mg<sup>2+</sup>-binding domain. The protein is a  
21 stable homodimer in solution mediated via reciprocal contacts between an  $\alpha$ -helical hairpin,  
22 located within the superhelix domain and additional asymmetrical contacts between the ends  
23 of the short arm of each L-shaped protomer. The dimer resembles U-shape with a crossbar  
24 formed from the hairpin of each partner. *C. difficile* binding is Mg<sup>2+</sup>-dependent. CDHS-1  
25 could not infect a *C. difficile* S-layer mutant suggesting the bacterial receptors are within the  
26 S-layer. These findings provide novel insights into phage biology and extend our knowledge  
27 of RBPs.

28 **Introduction**

29 *Clostridium difficile* is an anaerobic, Gram-positive, spore-forming bacterium responsible for  
30 gastrointestinal disease in humans (Vardakas *et al.*, 2012). *C. difficile* infection (CDI) is often  
31 associated with antibiotic treatment and disruption of the host gut microbiota that allows  
32 overgrowth of *C. difficile* (Burke and Lamont, 2014; Hargreaves and Clokie, 2014). CDI  
33 treatment is problematic because *C. difficile* is naturally resistant to most antibiotics.  
34 Alternative treatments for CDI are urgently required (Nale *et al.*, 2016). One such alternative  
35 is bacteriophages (phages), viruses that kill bacteria. Phages are often species-specific and  
36 consequently cause minimal disruption to the gut microbiota, yet they can self-amplify at the  
37 infection site. Phage therapy against CDI has been used successfully in animal models to  
38 reduce bacterial carriage and increase survival time following infection (Nale *et al.*, 2016).

39 For phage therapeutic development it is important to have a detailed understanding of the  
40 physical interactions between phages and their bacterial hosts and yet this is only known  
41 about for a relatively small number of phages. Not knowing the phage receptor binding

42 proteins limits our ability to develop phages as therapeutics in an informed way. As there is  
43 so little structural similarity between different phages the only robust way to identify RPBs is  
44 by using biochemical and structural approaches.

45 The infection process starts when phages attach to one or more receptor on the bacterial cell,  
46 and penetrate the cell membranes (Rakhuba *et al.*, 2010). This attachment process is mediated  
47 via interactions between proteins located at the end of the phage tail called receptor binding  
48 proteins (RBPs) and ligands (receptors) on the bacterial surface (Mahony and van Sinderen,  
49 2012).

50 Phage RBPs are structurally diverse, and many bacterial cell surface molecules can act as  
51 receptors including surface proteins, carbohydrates, or wall teichoic acids (Dowah and  
52 Clokie, 2018). RBPs are vital determinants of phage host range (Le *et al.*, 2013) and knowing  
53 what they are and how they work can inform novel diagnostics and therapeutics (Waseh *et*  
54 *al.*, 2010; Javed *et al.*, 2013; Simpson, Sacher and Szymanski, 2016).

55 Although sequence similarity is often limited between phages, comparative genomic analysis  
56 of phages infecting Gram-positive bacteria including *B. subtilis*, *L. lactis*, *L. monocytogenes*  
57 and *S. aureus*, previously revealed that genes encoding phage tail proteins are often located in  
58 the tail module between *tmp* gene encoding the tape measure protein (TMP), and the holin  
59 and endolysin proteins (Bielmann *et al.*, 2015).

60 Relatively few phages that infect Firmicutes have known, well characterized RBPs and the  
61 best known examples are *Staphylococcus aureus* phage  $\phi$ 11 and *Listeria monocytogenes*  
62 phages A118 and P35 (Bielmann *et al.*, 2015; Li *et al.*, 2016). Despite the fact that several  
63 phages that target clinically relevant *C. difficile* strains have been isolated, (Shan *et al.*, 2012;  
64 Nale *et al.*, 2016) no studies have identified their RBPs and little information is known about  
65 their bacterial receptors.

66 Recently, Kirk *et al.* showed that the S-layer protein of *C. difficile* acts as a receptor for  
67 Avidocin-CDs (are genetically modified versions of natural R-type bacteriocins that  
68 specifically kill *C. difficile* ribotype 027 strains). The *C. difficile* specificity of Avidocin-CD  
69 was acquired from fusing RBP phages such phi-027b, phi-123, phi147 and phi-242.c1 and  
70 Phi-68.4 (Kirk *et al.*, 2017). There is no sequence similarity between these RBP and Gp22  
71 amino acid sequence.

72 Here we report the identification of the RBPs from CDHS-1 and describe its three-  
73 dimensional structure. CDHS-1 is particularly effective on the epidemic 027 ribotype (Nale *et*  
74 *al.*, 2016). The capsid diameter of CDHS-1 is (70nm), and the tail length is 270 nm (Nale *et*  
75 *al.*, 2016).

76 Gp22 represents a new class of RBPs; it is a stable homodimer consisting of an N-terminal L-  
77 shaped  $\alpha$ -helical superhelix domain and a C-terminal  $Mg^{2+}$ -binding domain. Protomers of the  
78 dimer are asymmetrical with the short arm of one polypeptide interacting with its partner.  
79 Additional reciprocal contacts are mediated via an  $\alpha$ -helical hairpin within the super helical  
80 domain. This is the first study to identify the structure of RBPs for a *C. difficile* phage, and  
81 the work provides a mechanistic understanding of host recognition for these phages. It also  
82 identifies a novel class of RBPs thus expanding our comprehension of 'Phage receptor  
83 binding protein space'.

84

## 85 **Results**

### 86 **Putative functions of the CDHS-1 tail proteins**

87 To identifying the RBP's of CDHS-1, the tail putative tail module was analysed using  
88 bioinformatic approaches. A sequence analysis of the *C. difficile* CDHS-1 tail module  
89 identified four open reading frames that encode putative tail proteins: Gp18, Gp19, Gp21, and  
90 Gp22. The functional role of these proteins was investigated using prediction tools HHpred  
91 and Phyre2. The *in-silico* analysis revealed that Gp18 is homologous to the Gp19.1 protein  
92 from the *B. Subtilis* SPP1 phage (Figure S1). This *B. subtilis* Gp19.1 is a distal tail protein  
93 (Dit) (PDB 2X 8K), that forms the central hub of the SPP1phage baseplate, therefore it is  
94 likely Gp18 is the CDHS-1 Dit.

95 The HHpred analysis showed that CDHS-1 Gp19 is homologous to the gp18 protein from *L.*  
96 *Monocytogenes* phage A118 (PDB 3GS9) (Figure S1) and the Gp 44 protein of enteric phage  
97 Mu which are all tail-associated lysin-like proteins (Tal). The main function of these proteins  
98 is to help phages inject their genetic material into the bacteria by degrading the peptidoglycan  
99 layer (Bielmann *et al.*, 2015). Consistent with this putative role, a CDHS-1 Gp19 blastp  
100 search revealed that this protein has an endopeptidase domain (residues 7- 378).

101 CDHS-1 gp21 is homologous with the N-terminal domain of the ORF48 Upper baseplate  
102 protein (BppU) (PDB 3UH8) of phage TP901-1 of *L. lactis* (Figure S1). This protein links the  
103 RBP to the central baseplate core of *Lactococcus* phage TP901-1 so likely has a similar role  
104 in CDHS-1.

105 Finally, *in silico* analysis of CDHS-1 Gp22 revealed that there was no similarity to any  
106 protein in the Protein Data Bank (PDB), thus rendering it of particular interest as the potential  
107 RBP for this page. Of note, the blastp search showed that Gp22 is almost identical to the  
108 putative tail fibre proteins of other *C. difficile* phages from the database such as PhiCD38.2  
109 and phiCD146 only have a single amino acid difference, and homologous to the putative tail  
110 proteins of other phages infecting *C. difficile* phiCD111 has 82% similarity. Interestingly, the  
111 C-terminal 89 residues are less well-conserved (64% match) than the N-terminal 529 residues  
112 (86% match) (Figure S2). This is a similar pattern to well-studied RBPs in Lactococcal  
113 phages: sk1, TP901-1, and bIL170 phages. In each case, the receptor-binding sites are within  
114 the C-terminal (non-conserved) region of RBPs.

### 115 **Phage neutralization using polyclonal antibodies**

116 To determine if Gp22 does indeed determine phage infection, polyclonal antibodies were  
117 raised against recombinant Gp22 proteins, and the ability of it to neutralize CDHS-1 infection  
118 of *C. difficile* strain CD105LC1 was investigated. The putative upper baseplate protein Gp21  
119 was also overexpressed as a control and to establish if it's position could be determined on  
120 the phage particle. Antibodies were added to the phages and used in neutralisation assays.  
121 However, pre-incubation with anti-gp22 serum led to complete inhibition of the phage  
122 infection, up to a dilution of 1:10000 (Figure 1b). Thus, Gp22 plays an essential role in phage  
123 CDHS-1 infection of *C. difficile*. In contrast, pre-incubation of anti-Gp21 serum with  
124 CDHS-1 had no impact on phage infection (Figure 1a).

125

126

## 127 **The interactions of recombinant Gp22 protein with CD105LC1 strain**

128 To confirm that Gp22 is the CDHS-1 RBP, *C. difficile* CD105LC1 cells were incubated with  
129 glutathione-Sepharose 4B beads (Biosciences, Sweden) coated with either GST alone or  
130 Gp22 tagged with GST, and viewed using confocal microscopy under phase contrast. The  
131 bacteria cells attached to beads coated with GST-tagged Gp22 (Figure 2 a) but not to the  
132 beads coated with GST alone (Figure 2 b), indicating that Gp22 binds directly to *C. difficile*.

133 When increasing concentrations of recombinant Gp22 were pre-incubated with *C. difficile*  
134 CD105LC1 strain and in the presence of CDHS-1 phage, the phage adsorption was inhibited  
135 in a dose-dependent manner (Figure 2 c). Thus, it is clear that the Gp22 blocks the binding  
136 site on *C. difficile* for phage CDHS-1, confirming that Gp22 is the RBP. Unfortunately,  
137 attempts to produce a truncated folded form of Gp22 containing only the C-terminal region  
138 were unsuccessful because the truncated Gp22 was consistently degraded during the  
139 purification process.

## 140 **TEM Localisation of Gp22 and Gp21**

141 To identify the precise locations of Gp22 and Gp21 on the phage particle, CDHS-1 was pre-  
142 incubated with rabbit anti-Gp21 and anti-Gp22 sera. This was incubated with goat anti-rabbit  
143 secondary antibodies coupled with 12 nm gold colloids. The gold-labelled antibodies were  
144 seen as black spots under TEM, showing the location of each protein on the phage particle.  
145 CDHS-1 was incubated with the pre-immune sera from the rabbits as a negative control. The  
146 TEM showed that Gp21 and Gp22 are located on the tail baseplate of CDHS-1 (Figure 3a and  
147 b). As expected, only a random scattering of black spots was observed on the grids containing  
148 CDHS-1 incubated with the pre-immune sera (Figure 3d and e).

## 149 **Structure of Gp22 (RBP)**

150 To determine the 3D structure of Gp22 protein. Selenomethionine-enriched Gp22 protein was  
151 produced in *E. coli* and purified by affinity and gel filtration chromatography. Gel filtration  
152 analysis shows that Gp22 is a stable dimer in solution with an apparent molecular mass of  
153 ~130 kDa (expected mass of a dimer 136.4 kDa) (Figure 4a). Crystals of selenomethionine-  
154 Gp22 were grown at pH 7 in buffer containing  $Mg^{2+}$ . The phases were solved by  
155 selenomethionine single-wavelength anomalous dispersion, and data diffracted to a 2.5 Å  
156 resolution (Table 1). Four copies of Gp22 were observed in the asymmetric unit, all forming  
157 dimers through association with adjacent polypeptides about the crystallographic symmetry  
158 axes. Each polypeptide comprises an N-terminal L-shaped  $\alpha$ -helical superhelix domain (529  
159 residues) and a C-terminal  $\beta$ -sandwich domain. The rod like stems of the L-shaped super  
160 helical domain is formed from short parallel  $\alpha$ -helices (each of nine residues) with three  
161 helices per turn. A hairpin-like structure formed from a coiled-coil of  $\alpha$ -helices (Figure 4b) is  
162 located in the middle of the superhelix.

163 The C-terminal domain contains two metal-binding sites. The coordination geometry is  
164 compatible with  $Mg^{2+}$  (Figure 4 d), present in the crystallization buffer. Potential  
165 coordination ligands include the side chains of Asp540, Gln602, and Asp605, and the main-  
166 chain carbonyl groups of Gly606 and Asp540 for one  $Mg^{2+}$  and the side chains of Asp540  
167 and Asp605 for the second, more peripheral  $Mg^{2+}$ . Caution is needed in assigning  
168 coordination ligands given that the data were only of medium resolution and only certain  
169 water molecules were observed in the density.

170

171 The total dimer interface covers 5523 Å<sup>2</sup> of surface, 2760 Å<sup>2</sup> from one polypeptide, and 2763  
172 Å<sup>2</sup> from its partner. It is formed interactions between the two α-helical hairpin-like structures  
173 that pack together to form a 4-helix bundle. Additional contacts are made between the N-  
174 terminal portions of each superhelix domain. These domains come into contact through  
175 rotation of the short arms of the L-shaped polypeptides (by ~60°) in opposite orientations  
176 relative to the α-helical hairpin, thus creating an asymmetrical interface (Figure 4 c).

### 177 **The role of Mg<sup>2+</sup> in CDHS-1 phage infection**

178 Many phages require divalent cations ions to infect their bacterial hosts. To determine if  
179 divalent cations have a role in the CDHS-1 infection, phage were added on a lawn of  
180 CD105LC1 in the presence of Mg<sup>2+</sup> and/or Ca<sup>2+</sup>. The data show that CDHS-1 was only able  
181 to infect when the Mg<sup>2+</sup> was present in the media (Figure S3).

### 182 **The S-layer proteins of *C. difficile* contain the receptors for CDHS-1**

183 To identify if S-layer proteins also act as phage receptors for CDHS-1, was added to lawns of  
184 the S-layer deficient mutant FM2.5. The CDHS-1 could not infect this strain (Figure 5 b),  
185 however, infection was observed when the wild type R2029 strain was used (Figure 5 a).  
186 Thus, the receptors for CDHS-1 do indeed, reside within the S-layer proteins.

### 187 **Discussion**

188 The recent identification of a plethora of novel and diverse phages has not been matched by  
189 an understanding of their biology or the way by which they interact with bacterial cells. The  
190 reason for this mismatch is that the time taken to obtain sequence data is significantly less  
191 than that needed for structural studies. Furthermore, this data is key as it will improve our  
192 understanding of phage bacterial interaction and more importantly is downstream exploitation  
193 of phage for therapeutic purposes (Dowah and Clokie, 2018). One of the challenges of the  
194 phage therapy applications is the development of various phage resistance mechanisms via  
195 the bacterial host. Among these mechanisms, prevention of phage adsorption and DNA  
196 injection, restriction enzymes, and CRISPR/Cas systems (Principi, Silvestri and Esposito,  
197 2019). one of particular interest here is the prevention of the phage attachment. And to  
198 overcome this mechanism is by incorporating several phages with different host-range  
199 specificities within a cocktail (Yang *et al.*, 2020). Therefore, understanding phage-host  
200 interactions on a molecular level is crucial prior to phage therapy applications.

201 The phage proteins that mediate the bacterial phage interaction and determine phage  
202 specificity are the RBPs. They have been characterized for several phages that target Gram  
203 negative bacteria. This includes the *E. coli* T4, whose RBPs are well describe and  
204 understood (Mahony and van Sinderen, 2012) in addition to, lambda phage and T5 phage  
205 that have a monomeric RBP (Ppb5) that binds to the *E. coli* receptor FhuA. Pb5 exhibits an  
206 elongated shape in solution, and it is unlike any protein of known structure (Flayhan *et al.*,  
207 2012; Goulet *et al.*, 2020).

208 Relatively little is known about RBP's from phages that infect Gram-positive bacteria and  
209 less still for those that target *C. difficile*. The other RBPs from Siphoviruses that target Gram-  
210 positive bacteria that have been determined are ORF18 of phage P2, ORF 49 of phage  
211 Tp901-1, ORF20 from phage1358, ORF20 of bIL170, and ORF53 from Tuc2009 all of

212 which infect *L. lactis* (Dunne *et al.*, 2018) and Gp 45 from phage  $\phi$ 11 that infects *S. aureus*  
213 (Koç *et al.*, 2016). all known structures here, form trimeric complexes, with each monomer  
214 having a modular organization consisting of head, neck, and stem/shoulder domains, in  
215 which the shoulder domain contains the binding site for the bacterial host (Sciara *et al.*, 2008;  
216 Koç *et al.*, 2016; Dunne *et al.*, 2018).

217 The amino acid sequence comparison of those structurally determined RBPs reveal that, the  
218 RBPs of bIL170 and P2 phages are 89% identical at the N-terminal level and less identity  
219 found at C-terminal level which explained the diversity of the host range for the two phages  
220 (Dowah and Clokie, 2018). On the other hand, RBPs of phage  $\phi$ 11 infecting *S. aureus*  
221 organised in three parts too “stem”, a “platform” and a “tower”. Only the first 30 amino-acids  
222 of the stem resemble those of phages TP901-1 or Tuc2009 (Koç *et al.*, 2016).

223 Here we demonstrated that gp22 is the RBP for the Siphovirus CDHS-1 and that this protein  
224 is essential for a successful infection of *C. difficile*. This is the first description of a RBP of a  
225 phage that infects *C. difficile*. Notably, CDHS-1, infects the virulent strain 027 (Nale *et al.*,  
226 2016) so could potentially be developed as a novel antibiotic-independent therapeutic. the  
227 structure of Gp22 reported here is unlike any previously structurally described RBPs, and  
228 thus represents a new class of receptor binding proteins.

229 Gp22 is a U-shaped homodimer stabilised via a central crossbar formed from a four-helix  
230 bundle. Each subunit comprises an N-terminal L-shaped  $\alpha$ -helical superhelix domain, and a  
231 C-terminal  $\beta$ -sandwich domain. The protomers of each dimer are asymmetrical with the short  
232 arms rotated in opposite orientations to form the base of the U-shape. Such asymmetry is not  
233 uncommon in phage proteins. Gp22 was crystallised in the presence of  $Mg^{2+}$  two cations,  
234 compatible with  $Mg^{2+}$  are bound at the tip of the  $\beta$ -sandwich domain. This likely explains the  
235 observation that, infection by CDHS-1 is also  $Mg^{2+}$ -dependent.

236 We showed that CDHS-1 could not infect the S-layer mutant FM2.5 when CDHS-1 was  
237 spotted on the strain, indicating that the CDHS-1 receptors within the S-layer. The majority  
238 of phages infecting Gram-positive bacteria and have their receptors on the surface of the  
239 bacteria being identified have receptors within two main components on the surface of Gram  
240 positive bacteria; teichoic acid or peptidoglycan (Bertozzi Silva, Storms and Sauvageau,  
241 2016). To date, no work has been done to indicate that the S-layer proteins may act as phage  
242 receptors on *C. difficile*.

243 This is the first study to identify the RBP from phages that infect *C. difficile*. The results  
244 obtained from this study establishes a solid basis for understanding how phage attaches to *C.*  
245 *difficile*. Furthermore, the development of sensitive affinity-based infection diagnostics and  
246 therapeutics for this organism. Furthermore, a similar study ongoing for myoviruses that  
247 infect *C. difficile*.

248 Importantly this work changes the paradigm of existing phage receptor binding proteins  
249 having a specific structure, one of the key messages from the study is that there are likely to  
250 be completely novel classes of receptor binding proteins in existence which can only be  
251 identified through this type of biochemical and structural biological approaches. Interestingly,  
252 performing such structural studies was not trivial, Gp22 was quick to form a crystal however  
253 to produce a high-quality crystal that are able to diffract at a high resolution, it took extended  
254 period of time (3 years) with various optimisation steps. The key steps that influence Gp22  
255 crystals to diffract well were using, The BCS (Basic Chemical Space) Screen, Additive  
256 screen and seeding technique.

257

258

## 259 **Methods:**

### 260 **Bacteria, phages, and culturing**

261 The *C. difficile* strain used in this study for propagating phage CDHS-1 is CD105LC1 of  
262 ribotype 027, in addition to CDR20291 of ribotype 027, They both were from our Laboratory  
263 collections, and they have been previously described in detail (Nale *et al.*, 2016; Thanki *et*  
264 *al.*, 2018). Moreover, FM2.5 SlpA knock out (truncate SlpA at a site N-terminal to the post-  
265 translational cleavage site and, thereby, prevent the formation of an S-layer) derivative of  
266 R20291. *C. difficile* culturing and phage propagation were carried out according to a  
267 previously published protocol (Shan *et al.*, 2012). Briefly, *C. difficile* was grown under  
268 anaerobic conditions (10% H<sub>2</sub>, 5% CO<sub>2</sub>, and 85% N<sub>2</sub>) on Brain-Heart Infusion (BHI, Oxoid,  
269 Basingstoke, UK) 1% agar plates, supplemented with 7% defibrinated horse blood (DHB,  
270 TCS Biosciences Ltd., Buckingham, UK) for 24 hours at 37°C. Liquid cultures were prepared  
271 by taking a single colony from the blood agar plate and then inoculated in a bijou tube  
272 containing 5 ml of Fastidious Anaerobic Broth (FAB; BioConnections, Kynpersley, UK). The  
273 liquid cultures were left to grow anaerobically overnight. Then, 500 µl of overnight FAB  
274 culture was inoculated into 50 ml of pre-reduced BHI and incubated until an OD<sub>550</sub> of 0.2  
275 was reached. Subsequently, 500 µl of phage CDHS-1 was added to the culture and incubated  
276 for an additional 24 hours, followed by centrifuging at 3,400-x g for 10 min. The resulting  
277 supernatant was filtered using a 0.22 µm filter, and the phage titer in the filtrate was  
278 determined using a spot test assay according to a peer-reviewed protocol (Nale *et al.*, 2016).

### 279 **Protein Expression and Purification of the baseplate proteins (gp21 & gp22) for CDHS- 280 1 phage**

281 DNA extraction was carried out as previously described (Nale *et al.*, 2016). Post DNA  
282 extraction, both *gp21* & *gp22* were amplified using PCR. The primers used for the PCR are;  
283 5- GTGATAAATTTGAGAGATAG-3 and 5-TTAACTCACCTCTTCTTTTATTTC-3  
284 targeting

285 *gp21* gene, and 5-TACTTCCAATCCATGAGTTGGGCGGAGACATACAAAG-3 and 5-  
286 TATCCACCTTTACTGTCATTAAATTGCTTGATACATTGCGTAA-3 to amplify *gp22*  
287 gene. Then, the amplified genes (*gp21* and *gp22*) were cloned into pET-based expression  
288 plasmids with the help of the cloning service (PROTEX) based at the University of Leicester.  
289 The resulting plasmids were used to transform *E. coli* BL21 (DE3) and an established  
290 protocol of protein expression in *E. coli* using isopropyl β-D-1-thiogalactopyranoside (IPTG)  
291 as inducer was followed (Campanacci *et al.*, 2010). The proteins were purified using Affinity  
292 chromatography purification on Glutathione Sepharose 4B beads affinity column (GE  
293 Healthcare). After that, the proteins were further purified by gel filtration on 200 16/60  
294 columns (GE Healthcare). In 20 mM Tris pH 7.5, 20 mM NaCl and, then concentrated by  
295 filtration using a 10- kDa molecular mass cut off the membrane (Amicon) before further  
296 usage.

### 297 **Phage Neutralisation**

298 To determine which phage CDHS-1 tail protein was responsible for binding to *C. difficile*, the  
299 purified gp21 and gp22 proteins were sent to the Eurogenetec Company (Brussels, Belgium)  
300 for the generation of polyclonal antibodies. The resulting anti-serum antibodies were used in  
301 a 'phage neutralization test to determine which of these anti-sera would be able to neutralize

302 the phage infection. The assay was carried out according to a published protocol (Li *et al.*,  
303 2016). Briefly, each antibody serum was diluted using SM buffer (10 mM NaCl, 8 mM,  
304 MgSO<sub>4</sub>·7H<sub>2</sub>O, and 50 mM Tris-HCL pH 7.5), into 1:10, 1:100, 1:1000 and 1:10000. Then  
305 10<sup>5</sup> phage CDHS-1 was added to each of the dilutions mentioned above. The mixture was  
306 incubated for 20 minutes at 37 °C. Then the mixture was serially diluted using SM buffer and  
307 spotted on the lawn of the *C. difficile* strain used.

### 308 **The interaction of gp22 protein with CD105LC1 strain**

309 To determine if the recombinant gp22 interacts with CD105LC1, an adsorption inhibition  
310 assay was performed as in (Thanki *et al.*, 2018). Briefly, a strain grown in anaerobic  
311 conditions. Phage CDHS-1 (10<sup>7</sup> PFU/ml) and different concentration of gp22 proteins of  
312 400µg, 200µg, 50µg, 0µg were added into CD105LC1 culture with an OD<sub>600</sub> of around 0.2,  
313 respectively. After an incubation time of 30 minutes at 37 °C, a spot test was carried out to  
314 determine the phage titer. To visualize the direct interaction between gp22 and CD105LC1  
315 Glutathione Sepharose 4B beads coated with either GST-tagged gp22 protein or only GST.  
316 Then they were mixed with CD105LC1 cultures and incubated at 4°C for 30 minutes before  
317 confocal microscope. This assay was done as described in (Stevens *et al.*, 2005).

### 318 **Immune gold labeling**

319 Immuno-gold labeling was carried out to localize the phage tail protein gp21 and gp22 on the  
320 phage particle. The assay was done as described previously with slight modification (Li *et al.*,  
321 2016). In an Eppendorf tube, each antibody serum was diluted 1:100 using SM buffer and  
322 then incubated with 10<sup>9</sup> PFU/ml CDHS-1 phage for 20 minutes at room temperature. Then  
323 the mixture was applied to glow discharged, carbon-coated grids followed by grid washing  
324 with SM buffer for 10 minutes. Then a 1:30 diluted goat anti-rabbit IgG coupled with 12 nm  
325 gold colloids (Dianova, Hamburg) was added onto the grids and left for 20 minutes. Finally,  
326 the grids were negatively stained with 1% (w/v) uranyl acetate before examination under the  
327 TEM.

### 328 **Gp22 crystallization**

329 To determine gp22 structure, selenomethionine labeling was performed using the inhibition  
330 of the methionine pathway (Doublíe, 2007).

331 Briefly, *E. coli* BL21 (DE3) was grown overnight in 5 ml of LB. Then the cells were  
332 centrifuged for 5 minutes at 1300 x g. After that, the pellet was resuspended in 1 ml of M9  
333 (Molecular Dimensions, UK) (M9 prepared according to the manufacturer's guide) and added  
334 to 1 L of the same medium (M9). After this, the cells were left to grow in a 37 °C shaking  
335 incubator until they reached OD<sub>600</sub> of 0.2, then amino acids were added; lysine,  
336 phenylalanine, and threonine at a final concentration of 100 mg/ml, and isoleucine, leucine,  
337 and valine at a final concentration of 50 mg/ml. Thereafter, the cells were grown until an  
338 optical density OD<sub>600</sub> of 0.4 at 37°C, then IPTG was added at a concentration of 0.5 mM;  
339 finally, cells were incubated at 17°C overnight. Selenomethionine-gp22 protein purified as  
340 described above.

341 The purified Selenomethionine-gp22 protein was crystallized using the sitting-drop vapour  
342 diffusion technique. The crystals were grown in 0.1M HEPES pH 7, containing 0.05M  
343 ammonium acetate, 0.15M magnesium sulphate heptahydrate, 12% PEG smear medium, and  
344 4% acetone. Then the crystals were transferred to a reservoir solution containing 30 %  
345 glycerol as a cryo-protectant before freezing in liquid nitrogen. The diffraction data were  
346 collected from the Diamond light source.



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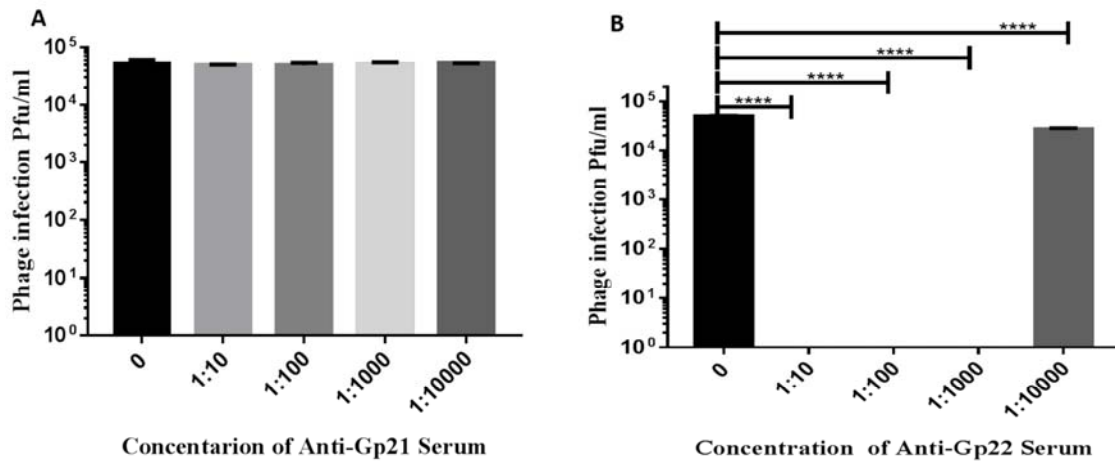
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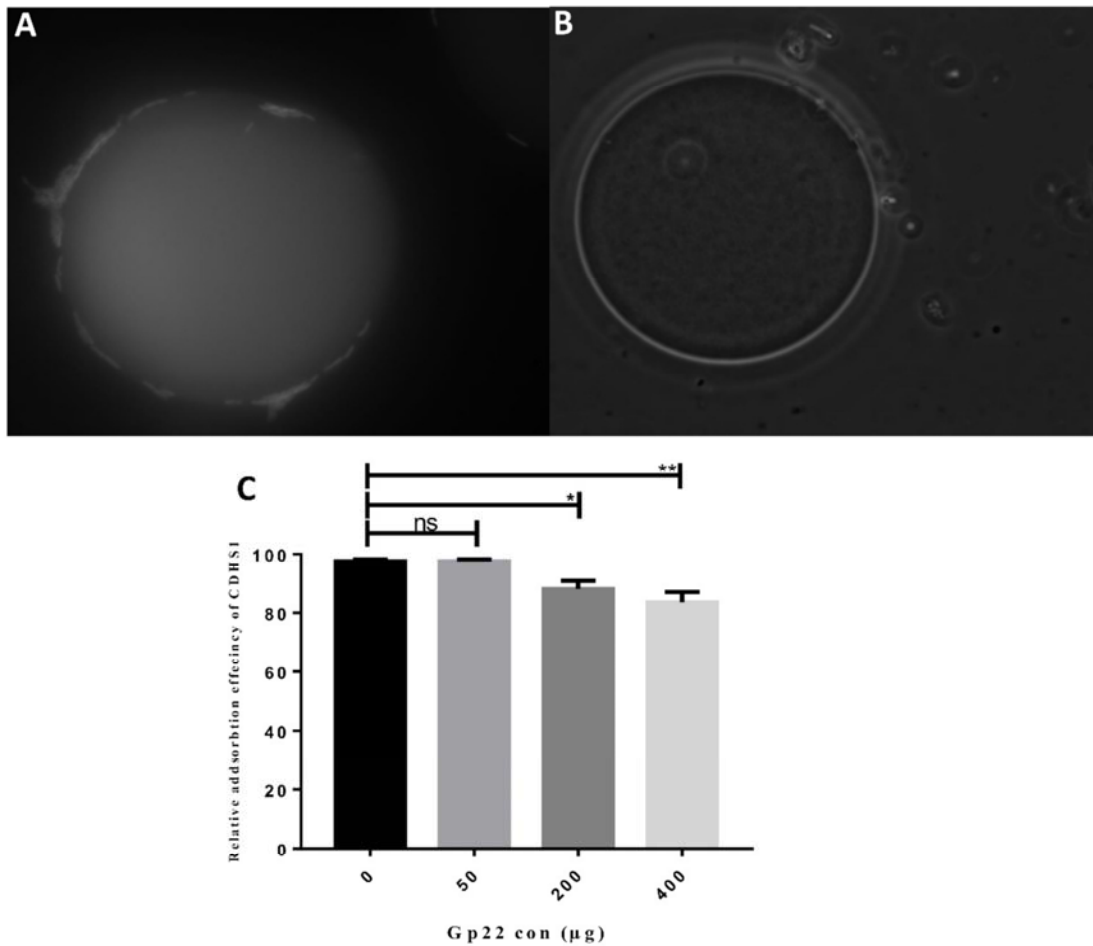
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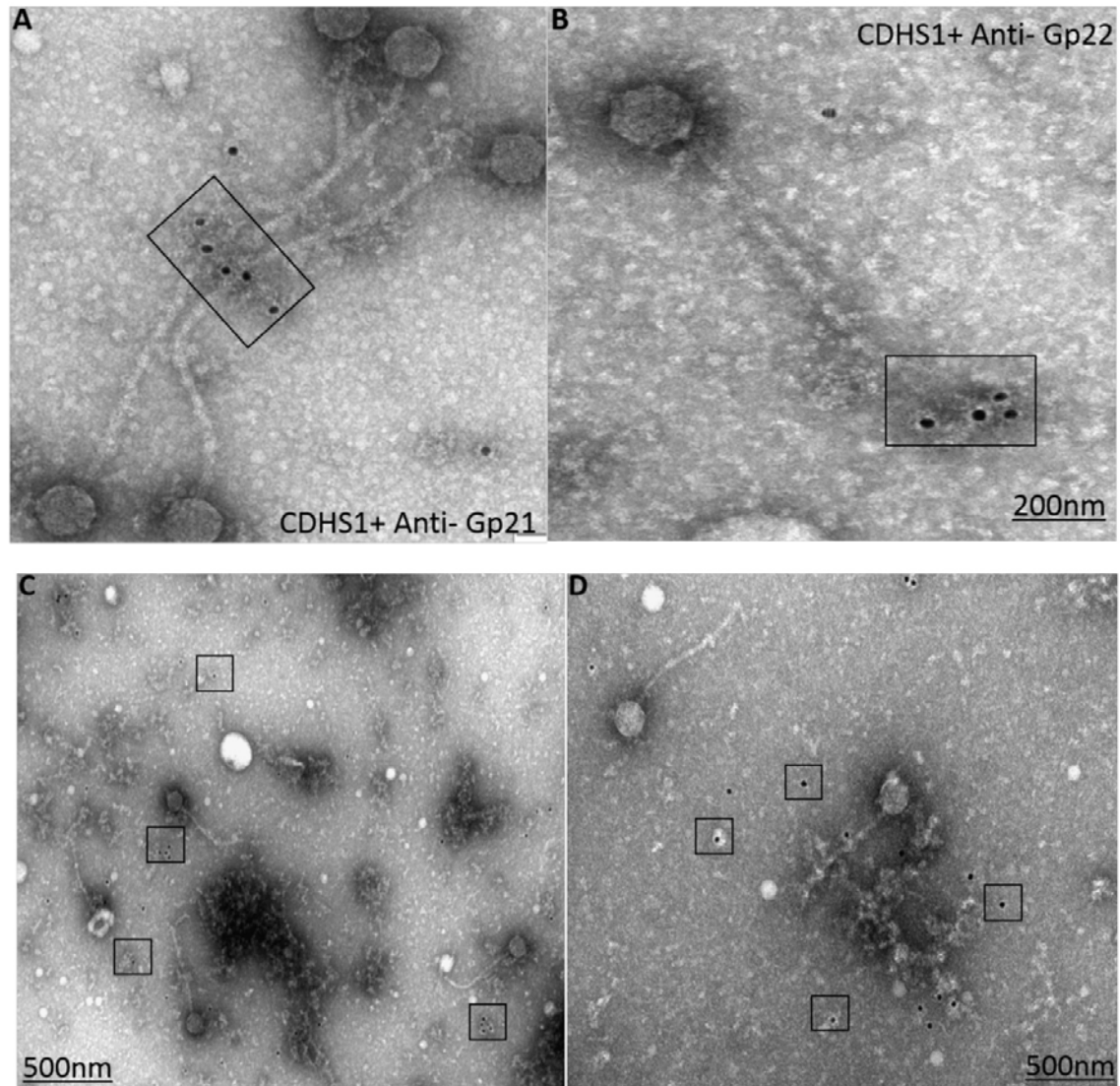
**Figure 1: The role of gp21 and gp22 proteins in CDHS1 attachment:**

CDHS1 was pre-incubated with different polyclonal antibodies against the tail proteins (gp21 and gp22). The phage was then tested for the ability to infect CD105LC1 strain using spot tests with serial 10-fold dilutions. (A) and (B) represent the plaque-forming units (Pfu/ml) of CDHS1 on CD105LC1 after incubation with different concentration of polyclonal antibody against gp21 and gp22 respectively. The result in (A) shows no significant differences between the positive control (gray column) and the negative control (black column) when anti-gp21 was used. (B) Represents the inhibition of phage CDHS1 infection on CD105LC1 strain, using different concentrations of anti-gp22 serum. The assay was conducted with three biological repeats each with three technical repeats. Error bar represent means  $\pm$  standard deviations (SD, n = 3). Statistical differences calculated by two-way ANOVA.



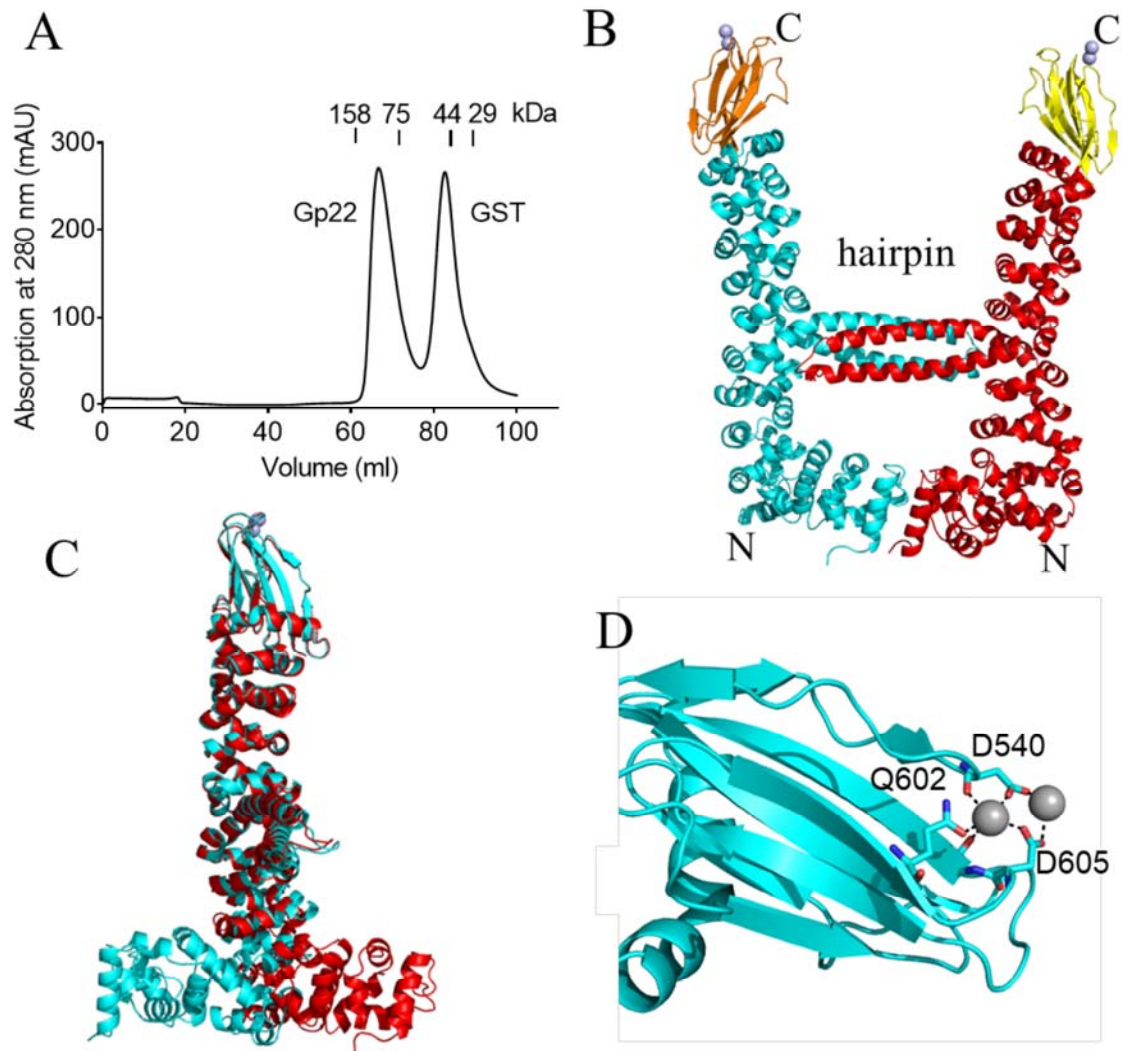
**Figure 2: The attachment of gp22 protein with CD105LC1 strain.**

(A) is a Confocal microscope image taken post-incubation of CD105LC1 strain with the Glutathione sepharose beads coated with gp22 protein tagged with GST. The result shows that the CD105LC1 cells attached to the beads coated with gp22 tagged with GST and indicates the gp22 protein has role in phage binding with *C. difficile*. As a negative control, the CD105LC1 cells pre-incubated with Glutathione sepharose beads coated with GST only and the result shows no attachment in image (B). (C) is a Dose-dependent inhibition of CDHS1 adsorption with recombinant gp22.



**Figure 3: Immunogold labelling of tail proteins, gp21 and gp22.**

Transmission electron microscopy (TEM) images of negatively stained phage CDHS1 after immunogold labelling with Anti- gp21 serum (A) and Anti -gp22 serum (B). (C) and (D) are the negative control (pre-immune sera for each antibody raised).



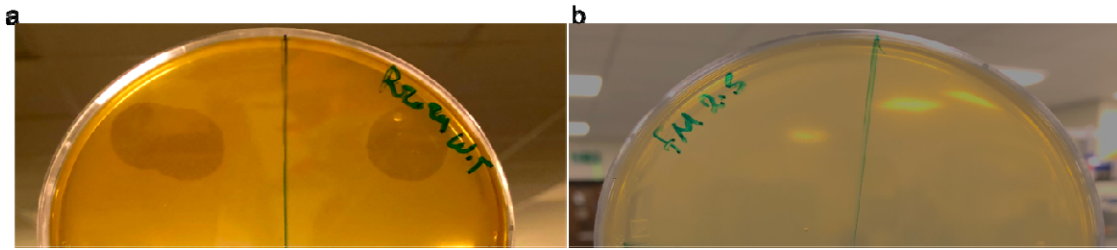
**Figure 4 Gp22 is a stable homodimer.** A, gel filtration of the gp22-GST fusion following cleavage by TEV protease. gp22 elutes with an apparent molecular mass of ~130 kDa from a Superdex 200 16/60 column based on the elution of molecular mass standards indicating that it is a dimer (monomer molecular mass = 68.4 kDa). GST is also dimeric as expected with a molecular mass of ~50 kDa B, the crystal structure of the gp22 dimer. The  $\alpha$ -helical superhelix domains are in red, cyan, and the  $\beta$ -sandwich domains in yellow and orange. N- And C- termini are indicated C, overlay of the gp22 polypeptides of the dimer. The  $\beta$ -sandwich domains and the C-terminal portions of the superhelix domains superpose closely, however the N-terminal portions are rotated by  $\sim 60^\circ$  in opposite directions. D, the  $\beta$ -sandwich domain showing bound  $Mg^{2+}$ . Putative coordination ligands are indicated.

**Table 1. Data collection and refinement statistics.**

Data collection	
Beamline	Diamond Light Source I03
Wavelength, Å	0.9793
Space group	P 2 <sub>1</sub> 2 <sub>1</sub> 2
a, b, c, Å	178.2, 227.5, 114.9
$\alpha$ , $\beta$ , $\gamma$ , °	90, 90, 90
Resolution, Å	140.3 - 2.52 (2.82 - 2.52)
No. reflections	104702 (5235)
R <sub>sym</sub>	0.167 (1.07)
CC(1/2)	1.0 (0.7)
I/ $\delta$ I	9.1 (1.6)
Completeness	95.8 (73.3)
Redundancy	6.6 (7.1)
Refinement	
Resolution, Å	140.3 - 2.52 (2.61 - 2.52)
No. reflections	104684 (291)
Multiplicity	6.6 (7.1)
R <sub>work</sub> /R <sub>free</sub>	18.8/22.8
No. atoms	18877
Protein	18738
Ligands	8
Water	133
B-factors, Å <sup>2</sup>	46.4
Protein	45.7
Ligand	65.8
Water	40.5
RMS (bonds) Å	0.012
RMS (angles) °	1.45

Statistics for the highest-resolution shell are shown in parentheses.





**Figure 5: CDHS1 phage infection to CDR20291 and FM2.5 SlpA knock out derivative of R20291:** CDHS1 Spotted on a lawn of CDR20291(a) and FM2.5 (b)strains, CDHS1 was not able to infect the FM2.5 (b). As it did when spotted on the wild type CDR20291 (a). That is indicate that receptors that CDHS1 targeted are within the S-layer protein of CDR2029.