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Targeting Vibrio Cholerae host-bacterial interactions to prevent endemic and epidemic cholera

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Targeting Vibrio Cholerae host-bacterial interactions to prevent endemic and epidemic cholera.

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Master of Medical Research
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Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

(Signed)______***Ronald Alexander Coetzee***_______

Page of acknowledgement

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Abstract

Cholera, caused by the Gram-negative bacterium *Vibrio cholerae*, remains a significant world-wide public health issue, causing severe and often fatal diarrhoeal illness in humans. *V. cholerae* biofilm has been suggested to play a critical role in the pathogenesis and disease transmission. However, the role of intestinal glycans in biofilm formation during infection, as well as their role in adhesion of *V. cholerae* to host cells, colonization and virulence, remains poorly understood. In this study we explored the involvement of Vibrio polysaccharide (VPS), Lipopolysaccharide (LPS) and biofilm matrix proteins in the presence of monosaccharides. Glycan array analysis of multiple *V. cholerae* strains revealed that common glycans interacted with bacterial cells, such as gangliosides (GM1), blood group antigens and Lewis antigens for both the wild type strain and its rugose variant, a VPS overexpressing variant of the wild type. The VPS, LPS and biofilm matrix protein mutants all had greatly reduced ability to interact with these glycans. Interestingly, only the rugose variant and VPS mutant strains showed the ability to bind to blood group H and P antigens, while biofilm matrix protein and LPS mutants lost these bindings, indicating that an overproduction of VPS may allow for additional interactions with the host cell. Some of the important glycans that had the ability to interact with the *V. cholerae* cells were galactose, mannose, glucosamine, fucose, and in addition, mucin. Biofilm assays showed that galactose had an inhibitory effect on the biofilm formation by the rugose variant and the LPS mutant, while it had a stimulatory effect on the biofilm formation of the biofilm matrix protein mutant. Mannose was revealed to have an inhibitory effect on the biofilm formation of the VPS and LPS mutants, while a stimulatory effect was noticed on the biofilm formation of the biofilm matrix protein mutants. Glucosamine had an inhibitory effect on the biofilm formation by the wild type strain and a stimulatory effect on the biofilm formation of the biofilm matrix protein mutant. Fucose and mucin were shown to have an inhibitory effect on the biofilm formation by the wild type strain, its rugose variant, the VPS, LPS and biofilm matrix protein mutants. In addition, the level of adhesion displayed by the wild type, its rugose variant, and its VPS, LPS and biofilm matrix protein mutant strains to Caco-2 cells were investigated. The results revealed severe adhesion deficiencies in the VPS, LPS and biofilm matrix protein mutant strains. Environmental and clinical strains were also tested for adhesion with Caco-2 cells but yielded no discernible pattern regarding adhesion capabilities. One environmental strain and one clinical strain displayed severe adhesion deficiencies, while the other environmental and clinical strain displayed a greater adhesion than the wild type. Biofilm assays revealed that, when compared to the biofilm formation of the smooth wild type strain, all environmental and clinical strains tested had an increase in biofilm formation. The study and testing of more environmental and clinical strains may reveal an association in biofilm formation between environmental and disease settings.

Chapter 1

Introduction

Cholera, caused by the Gram-negative bacterium *Vibrio Cholerae (V. cholerae)*, remains a significant world-wide public health issue, causing severe and often fatal diarrhoeal illness in humans. *V. cholerae* causes approximated 4 million cases and 100 000 deaths world-wide annually (Lekshmi et al., 2018) and the rates are rising due to natural disasters and the effects of climate change. *V. cholerae* incidences peak during times of high monsoonal rainfall, which often lead to flooding (Shackleton et al., 2023). A single flood in West Bengal caused over 16 000 *V. cholerae* cases and 276 deaths (Hadeed et al., 2022). Floods can also damage existing water infrastructure and spread *V. cholerae* to new areas, thus exacerbating the rates of the disease (Gelormini et al., 2023).

The mainstay of current therapy is the supportive management of secretory diarrhoea with oral and intravenous fluids, with escalation to antimicrobials and antibiotics, to which there is already high levels of resistance in the community. Therefore, it is vital that better strategies for prophylaxis and treatment be developed to prevent the significant morbidity and mortality in future outbreaks.

Since it is already known that VPS, LPS and the biofilm matrix proteins RmbA, RmbC and Bap1 play crucial roles in the development of *V. cholerae* biofilms (Staropoli & Alon, 2000; Fong et al. 2010; Mohammad Pour Ghazi & Gargari, 2016; Teschle et al. 2022; Huang et al. 2023), it was speculated that mutant strains missing these genes will produce less biofilm when compared to normal wild type strains. These truncated strains may also bind to different or even fewer glycans, and as such might affect the aggregation and adhesion of *V. cholerae* cells (Busch et al. 2012; Leo et al. 2012; Thomas et al. 2018). Since the effect of different monosaccharides on the biofilm formation of these truncated strains are currently unknown, this study aims to explore these questions and hypothesis.

1.1 The epidemiology of *Vibrio cholerae*

V. cholerae is a Gram-negative bacterium responsible for the severe human diarrheal disease cholera. Cholera is characterized by voluminous watery diarrheas and vomiting, which may rapidly lead to hypovolemic shock, acidosis, and death, with an untreated case-fatality rate as high as 50% (Nadri et al., 2018). Seven recorded major cholera epidemics have been recorded since 1871, though the disease likely has an ancient association with humans. Although the development of oral rehydration (David, 2022) has reduced the treated case fatality rate substantially, cholera continues to impose an enormous global health burden (Im, 2019., Ali, 2015). One contributor to this is the high morbidity of cholera, especially in low socio economic areas and countries, where sanitation and clean drinking water is virtually non-existent (Gallandat, 2021). *V. cholerae* also remains in “hotspots”, spreading periodically to unaffected areas via river and water systems (Kayembe, 2021). From these aquatic reservoirs, *V. cholerae* spreads to populations of the natural host, humans, through contamination of water and food. This is achieved through a faecal-oral route (Ali, 2018).

Although patients are likely to survive infection due to rehydration therapy, the debilitating diarrhea characteristic of cholera continues for days even if the pathogen is cleared with antibiotics (Ganesan et al., 2020). With *V. cholerae* widely distributed in every major continent except North America and Australia, the bacterium mainly affects poor and developing countries. Thus, the health and economic burden placed upon these places are exacerbated by the deliberating effects of cholera, like straining weak, underfunded and already overburdened national health systems, skyrocketing the inaccurate reporting of cases, which drains valuable recourses, and limiting access to treatments and vaccines (Gallandat, 2021., Asadgol, 2020).

1.2 The spread and symptoms of *Vibrio cholerae*

When infection of *V. cholerae* occurs via oral digestion of contaminated water, it is usually the serotypes of O1 and O139 that are the most prevalent in epidemic cases. The bacterium produces cholera toxin (CT), which can cause watery diarrhea of such intensity that hypotensive shock and subsequent death can occur within 12 hours of the appearance of the first symptoms (Ganesan et al., 2020). Due to the ability of *V. cholerae* to spread rapidly via contaminated water sources and diarrhea from infected patients, numerous cases are usually reported in the same community. This high infection rate, combined with poor sanitation and close proximity can lead to subsequent epidemics (Gallandat, 2021). In poorer countries such as Yemen, the large number of people infected, and the severity of the disease caused by the O1 El Tor (7PET) lineage, has devastated the community, despite being susceptible to several common antibiotics used to treat cholera (Weill, 2019). The incubation period of *V. cholerae* can range from several hours to 5 days and is dependent on inoculum size (Nadri et al., 2018). The onset of the illness may be sudden, with profuse watery diarrhea, or there may be initial symptoms like abdominal discomfort and simple diarrhea. Mucus in the stool gives the ‘rice water’ appearance that is generally associated with cholera. Vomiting is often present, occurring a few hours after the onset of the diarrhea. In the most severe form, termed cholera gravis, the diarrheal rate may reach 500 to 1000 ml/h, leading to tachycardia, hypotension, and vascular collapse due to dehydration (Morris Jr, 2011). Severe dehydration can lead to death within hours of the onset of symptoms unless fluids and electrolytes are rapidly replaced.

1.3 The serogroups of *Vibrio cholerae*

Although *V. cholerae* is serologically diverse with more than 206 serogroups reported, only the O1 and O139 serogroups, as discussed above, have been reported to cause epidemic cholera . The first six cholera pandemics were all caused by the classical O1/O139 biotype *V. cholerae*, whereas the El Tor O1 biotype is responsible for the recent, seventh pandemic. These two *V. cholerae* biotypes differ considerably; El Tor strains generally cause a milder form of cholera than that caused by classical strains and apparently evolved as better survivors in the aquatic environment (Ramamurthy et al., 2020). Currently, El Tor strains are predominant everywhere that *V. cholerae* O139 can be found (Roy, 2005).

Between epidemics, *V. cholerae* resides in aquatic environments such as freshwater lakes and rivers, where these bacteria interact with various surfaces in the form of biofilms, which form an important survival mechanism (Silva & Benitez, 2016).

1.4 The transmission of *Vibrio cholerae*

It has been shown that *V. cholerae* has two established life cycles, an aquatic phase and a human intestinal phase (Vezzulli et al., 2008). *V. cholerae* remains in a non-culturable but viable phase when in an aquatic environment. It has been shown that the optimal growth conditions for *V. cholerae* in the aquatic phase are 15% salinity, 30^{0}C and a pH of 8.5 (Chowdhury et al., 2017). Epidemics are usually preceded by warmer temperatures and either higher rainfall amounts, or droughts (Codeco, 2001). This is believed to occur due to the increase the phytoplankton during the warmer months. Phytoplankton increase the pH of their surrounding environment, which makes conditions favourable for *V. cholerae*. Outbreaks have also been shown to be seasonal, with case numbers periodically peaking once or twice a year. Again, this is thought to be due to warmer temperatures and higher water levels, which brings with it an increased food supply for the bacterium (Asadgol, 2020). Rainfall also washes faeces into water systems, increasing the chance for *V. cholerae* contamination. (Codeco, 2001). This allows large outbreaks to occur with relative ease in communities with no alternative water supplies. With *V. cholerae* then continuously circulating through the population, new biotypes and mutations are constantly being formed in its aquatic reservoirs, thus ensuring that *V. cholerae* will not be eradicated in its natural environment (Chowdhury et al., 2017; Jutla et al., 2013).

When *V. cholerae* enters the human gut, the bacterium switches from its aquatic to human phase, first via reversible attachment, then non-reversible attachment (Vezzulli et al., 2008).

Recently, there has been a large surge in antibiotic resistance around the world (Anderson, G. G., & O'Toole, G. A., 2008; Ye et al., 2017). One of the reasons may be attributed to the increased disposal of antibiotics and non-degradable antimicrobials by humans into aquatic environments containing *V. cholerae* (Cabello, 2006). This causes the natural selection for more and more resistant bacteria (Vezzulli et al., 2008).

1.5 Virulence factors

The original definition of virulence has been the ability of a pathogen to cause death (Johnson, 2018). However, in recent years, the definition and factors encompassing virulence has expanded greatly. Today, virulence takes multiple factors into account, such as damage caused to the host, the amount of bacteria needed to damage the host, the level of mortality associated with the infection, the toxicity of the primary pathogen, the incubation time before the onset of symptoms occur and the communicability of the disease (Johnson, 2018., Gurtler, 2017). These categories together help to better quantify the Pathogenic Potential (PP) and the effect a pathogen might have on a host (Johnson, 2018).

There are many factors that affect the virulence of a pathogen, and historically, these have been seen as direct components of a pathogen to cause disease and/or death (Johnson, 2018). Recently, in addition to those mentioned above, molecules or structures that aid in immune evasion or modulate host defences for the pathogens replicate advantage, adherence factors that aid in cell adhesion, such as biofilms and bacterial capsules (Johnson, 2018. Gurtler, 2017).

1.5.1 Vibrio cholerae adhesion

So far, it is known that the organism attaches to the gut mucosa and forms microcolonies before it secretes known virulence factors such as cholera toxin (CT) and co-regulated adhesins, including co-regulated pilus (Garcia-Peñarrubia et al., 2002; Ramamurthy et al., 2020; Vezzulli et al., 2008). The pili bind the *V. cholerae* cells together to combat the shearing forces that may be experienced in the gut (Garcia-Peñarrubia et al., 2002). It mediates adherence between cells and microcolony formation, which is critical for biofilm formation and colonisation of the small intestine (Silva & Benitez, 2016). The initiation of this process is likely to occur by interaction of the organism via bacterial adhesins with glycosylated host cell surface molecules. However, the host and bacterial molecules involved, and the nature of such interactions remain largely undetermined.

Although the role of auto-aggregation is often poorly understood, it is believed to occur in response to external stress, such as nutrient depletion or oxidative stress. By clumping together, bacterial cells have a greater chance of reducing the environmental stress placed upon them and may even protect cells from the immune system (Thomas, 2018).

There are many factors that aid and mediate auto-agglutination. Gram positive and gram-negative bacteria utilize both pili and fimbriae to mediate auto-aggregation. These may include chaperone-usher (C-U) pathway pili, contractile type IV pili and curli fibers (Busch, 2012). Auto-transporter proteins, such as the trimeric auto-transporter adhesins (type Vc) and classical auto-transporters (type Va), may also have a part to play in Gram negative bacteria auto-aggregations (Thomas, 2018; Leo, 2012).

It is widely known that *V. cholerae* attaches to human intestinal cells before causing disease. After micro auto-aggregation occurs, *V. cholerae* cells reversibly bind to the surface of human epithelial cells (Yildiz, F. H., & Visick, K. L., 2009; Silva & Benitez, 2016). When the bacterium becomes established, the binding becomes irreversible. Quorum sensing then leads to exopolysaccharide secretion and biofilm formation. This leads to the creation of microcolonies, which leads to mature biofilms forming, allowing pathogenesis to occur (Wolska, 2016; Silva & Benitez, 2016).

The main virulence factor for *V. cholerae* cells is the cholera toxin (Boesveld et al., 2019). A 2 subunit toxin, the B-subunit binds to the ganglioside GM1 receptors, while the A subunit translocates through the membrane to cause disease (Silva & Benitez, 2016; Boesveld et al., 2019). Therefore, most cholera strains are non-invasive (Lencer, 2001).

1.5.2 Cholera toxin

Upon human colonization, the virulence of *V. cholerae* is due primarily to the production of cholera toxin (CT), which alters host cell signal transduction pathways and leads to cell damage and diarrhea, and the toxin coregulated pilus (TCP), which is critical for colonization of the intestinal epithelium. The actions of the toxin are well studied, and although not all mechanisms are fully understood there is considerable insight in its pathogenesis. There are 2 subunits to the toxin, Subunit A and Subunit B. The B-subunit binds to the ganglioside GM1 receptor of the human mucosa (Boesveld et al., 2019). Binding of the toxin requires that at least two of the five B-sub-units interact with the GM1 ganglioside. After binding, the A-subunit is translocated across the membrane, although how this is achieved is still unknown (Watanabe et al., 2018., Zingl, 2021). Translocation requires the reduction of the disulfide bond between the A1 and A2 subunits. It is speculated that the A2-subunit does not enter the cell (Banerjee, 2020). Once inside the cell the cholera toxin (CT) catalyses the transfer of the ADP-ribose moiety of NAD to the alpha-subunit of the Gs protein (Zingl, 2021). The Gs protein then activates adenylate cyclase, which in turn mediates the transformation of ATP to cyclic AMP (cAMP) (Watanabe et al., 2018). The cAMP is an intracellular messenger for a variety of cellular pathways, and activates a cAMP-dependant protein kinase A, leading to protein phosphorylation, altering of ion-transport (increased Cl ion secretion), and ultimately to diarrhea (Boesveld et al., 2019). The exact mechanism is not completely understood, and it is speculated that this mechanism involves prostaglandins and interactions with the enteric nervous system as well as the immune system.

1.5.3 Vibrio cholerae biofilms

One known key factor important for *V. cholerae*'s ability to cause epidemic disease is its ability to thrive in matrix-enclosed surface-associated communities, known as biofilms, as shown in Figure 1.5.3. *V. cholerae*, cells encase themselves in a self-producing matrix, which alters their growth rate and pathogenicity (Silva & Benitez, 2016). Humans ingest *V. cholerae* as part of the pathogen's normal faecal-oral transmission route. Cholera stools contain both free-living cells and biofilm-like aggregates of *V. cholerae*. In vivo-formed biofilms also increase *V. cholerae* infectivity, transmission, and environmental persistence (Corfù et al., 1992;

Halouska et al., 2014), and the removal of particles >20 µm 2 in diameter from water reduces cholera incidence by 48% (Johnson et al., 2017).

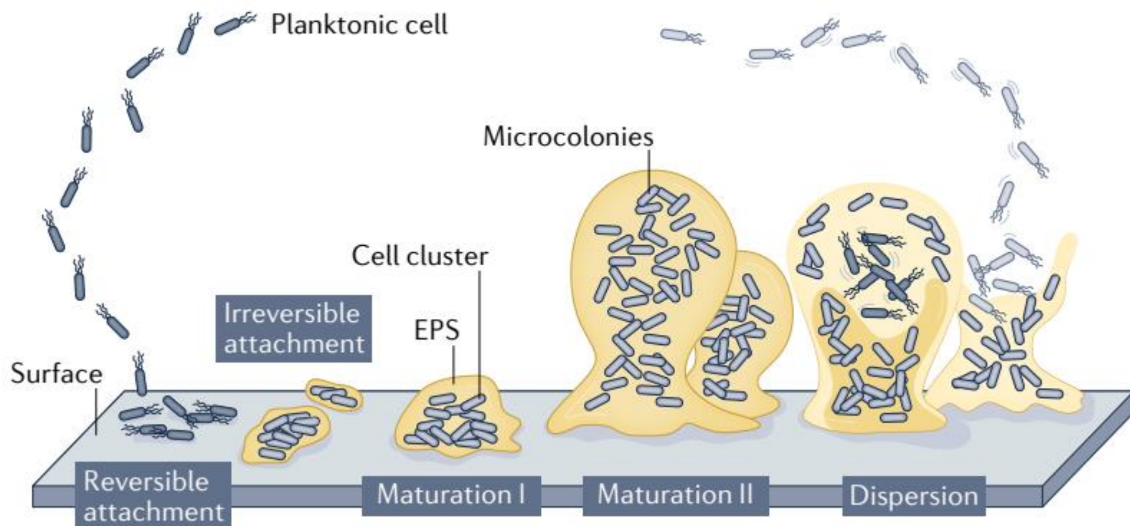

Figure 1.5.3: An overview of the biofilm formation process. The figure above describes the initially reversible formation, then irreversible formation of bacterial biofilm to the host surface. Bacteria initially attach to the host surface via flagellum or co-regulated pili. This results in the upregulation of biofilm matrix components. These clusters then mature into micro-colonies in a relatively safe and nutrient rich environment. Dispersion allows for the continuous spread of the pathogen, and the continuation of the cycle. EPS, extracellular polymeric substances. Sauer, 2022.

Biofilms have been shown to aid in the resistance of antibiotics and increase the transmission and pathogenicity of Cholera (Jose et al., 2017). Furthermore, *V. cholerae* biofilms have been shown to be hyper-infective, but disease progression of biofilm-mediated infections is still unclear. Therefore, it is critical to assess how both types of cells, planktonic and biofilm-encased, interact with human intestinal cells in order to cause disease (O'Toole, 2000).

Once *V. cholerae* cells have established themselves on the gut mucosa, they downregulate genes required for motility and upregulate genes required for biofilm production in a no-human model (Silva & Benitez, 2016). This is done via quorum sensing. Quorum sensing is a cell-to-cell communication process via the production, secretion and detection of auto-inducers, a type of extra-cellular signalling molecule (Papenfort & Bassler, 2016; Wolska, 2016). This enables bacteria to, as a collective unit, modify their behaviour and alter their global patterns of gene expression in response to changes in the cell density and species composition of the surrounding microbial community (Papenfort & Bassler, 2016). Cells then self-secrete a biofilm extracellular matrix around them, forming a barrier between them. Entry through this barrier is limited by diffusion and allows cells to transition into the stationary phase (Silva & Benitez, 2016). Thus, it is imperative to target the disease process before the formation of a stable biofilm, potentially inhibiting the formation of the biofilm and thus preventing disease (Gupta, 2016; Lekshmi et al., 2018).

1.6 Glycan components of *Vibrio cholerae*

V. cholerae causes disease by adhering to cell surface structures of the intestinal wall. These structures act as binding sites for pathogens and consist of sugar-rich molecules, glycans, which are sugars attached to proteins or fats. Thus, the understanding of pathogen-host interactions such as binding to cell surface structures of the intestinal wall is of critical importance.

1.6.1 Role of VPS in Vibrio cholerae

The biofilm matrix is predominantly formed from Vibrio exopolysaccharide (VPS), and it is known to be essential for biofilm formation (Staropoli & Alon, 2000., Fong , 2010). However, how VPS production during infection contributes to disease, is debated (Cha et al., 2019; Micciche et al., 2019). Since VPS is structured from many and varied glycans, its role in host-pathogen interactions and adhesion needs to be established.

The rugose variant is a VPS overexpressing variant of the A1552 smooth wild type strain, and it was in the rugose variant that VPS itself was first isolated. It was discovered that glucose, galactose, mannose and N-acetyl-glucosamine were some of the main components that make up VPS (Fong, 2010). It is produced shortly after the initial attachment and aids in the formation of the three-dimensional structure of the biofilm matrix (Teschler, 2015).

1.6.2 Role of LPS in Vibrio cholerae

Another important surface structure which is known to be important in *V. cholerae* infection and disease is the lipopolysaccharide (LPS), and strains lacking LPS are severely attenuated for virulence (Iredell et al., 1998; Mohammad Pour Ghazi & Gargari, 2016). LPS is abundant on the bacterial cell surface and determine which serotype a particular strain exhibits (e.g., serotype O1 El-Tor of epidemic strains). Since LPS is structured from many and varied glycans, its role in host-pathogen interactions and adhesion needs to be established.

A major component of the outer membrane of *V. cholerae* is LPS. The three main layers are the O-antigen, the core oligosaccharide and Lipid A (Nesper, 2001). The O-antigen is exposed to the outer environment, while the Lipid A section is buried in the outer membrane. The LPS is involved in creating a permeable layer for the movement of nutrients and protection against harmful molecules and substrates (Teschler, 2015).

1.6.3 Role of biofilm matrix proteins in Vibrio cholerae

There are 3 biofilm matrix proteins that are of particular importance in the formation and maintenance of *V. cholerae* biofilms: Rugosity and biofilm structure modulators A (RbmA), Rugosity and biofilm structure modulators C (RbmC) and its homolog Bap1 (Fong, 2007).

RbmA contains at least two fibronectin type III folds, which is critical for cell to cell and cell to biofilm adhesion (Teschler, 2015). Between these two folds is a linker segment, which forms a large groove, capable of accommodating VPS and other filamentous substrates, and a small grove, capable of accommodating LPS. This positions RbmA to function as a scaffolding protein, providing early elasticity and stability to the forming biofilm (Teschler, 2022).

Bap1, the homolog of RbmC, is produced and secreted near the cell surface and is thought to be involved in cell adhesion. Bap1 is mainly involved with adherence to lipids and abiotic surfaces. Bap1 is also thought to aid in maintaining pellicle strength and contribute to pellicle hydrophobicity (Teschler, 2015., Huang, 2023).

RbmC is a homolog to Bap1 and is secreted in specific locations near the cell surface. RbmC, together with VPS and Bap1, can form flexible envelopes around *V. cholerae* cells that grows as the cells divide (Teschler, 2022., Huang, 2023). RbmC is mainly involved in mediating host binding and adhesion.

RbmC was shown to be of critical importance for the incorporation of VPS into the biofilm (Teschler, 2015). Furthermore, *V. cholerae* cells that did not produce VPS were unable to accumulate RbmA, RbmC and Bap1 on or near their cell surfaces. Thus, the biofilm matrix is made up of a combination of RbmA, RbmC, Bap1 and VPS (Teschler, 2022, Huang, 2023).

1.7 Pathogen-host interactions and *Vibrio cholerae*

As shown in Figure 1.7, *V. cholerae* has complex interactions that bring it into contact with many different molecules and glycans. A few limited studies into associations of *V. cholerae* with host glycans post-infection indicated that bacterial antigens, such as cholera-toxin subunit B, can interact with human ganglioside GM1 (De et al., 2018; Holmgren et al., 1975). Vibrio chitin-binding proteins can bind chitin, and biofilm matrix protein RbmA, can bind galactose, fucose and sialic acid (De et al., 2018). Few other potential interactions with the host molecules, such as blood group antigens, have been suggested based on epidemiological and population studies (El-Hawiet et al., 2015; Lambert & Neuhaus, 1972; Tribble et al., 2010). However, little is known regarding the breadth or specificity of glycan interactions, important to the infection of the human host. Furthermore, nothing is known of glycan-glycan host-pathogen interactions of this organism, an important aspect of bacterial virulence. It was shown that other bacterium such as *Shigella flexneri, Haemophilus influenzae, Salmonella enterica and Campylobacter jejuni* can interact with high affinity with human host cells via glycan, and glycan-glycan interactions (Day et al., 2015). If similar results can be replicated for both epidemic and non-epidemic strains of *V. cholerae*, it will be the basis of future development for new and innovative strategies to combat cholera.

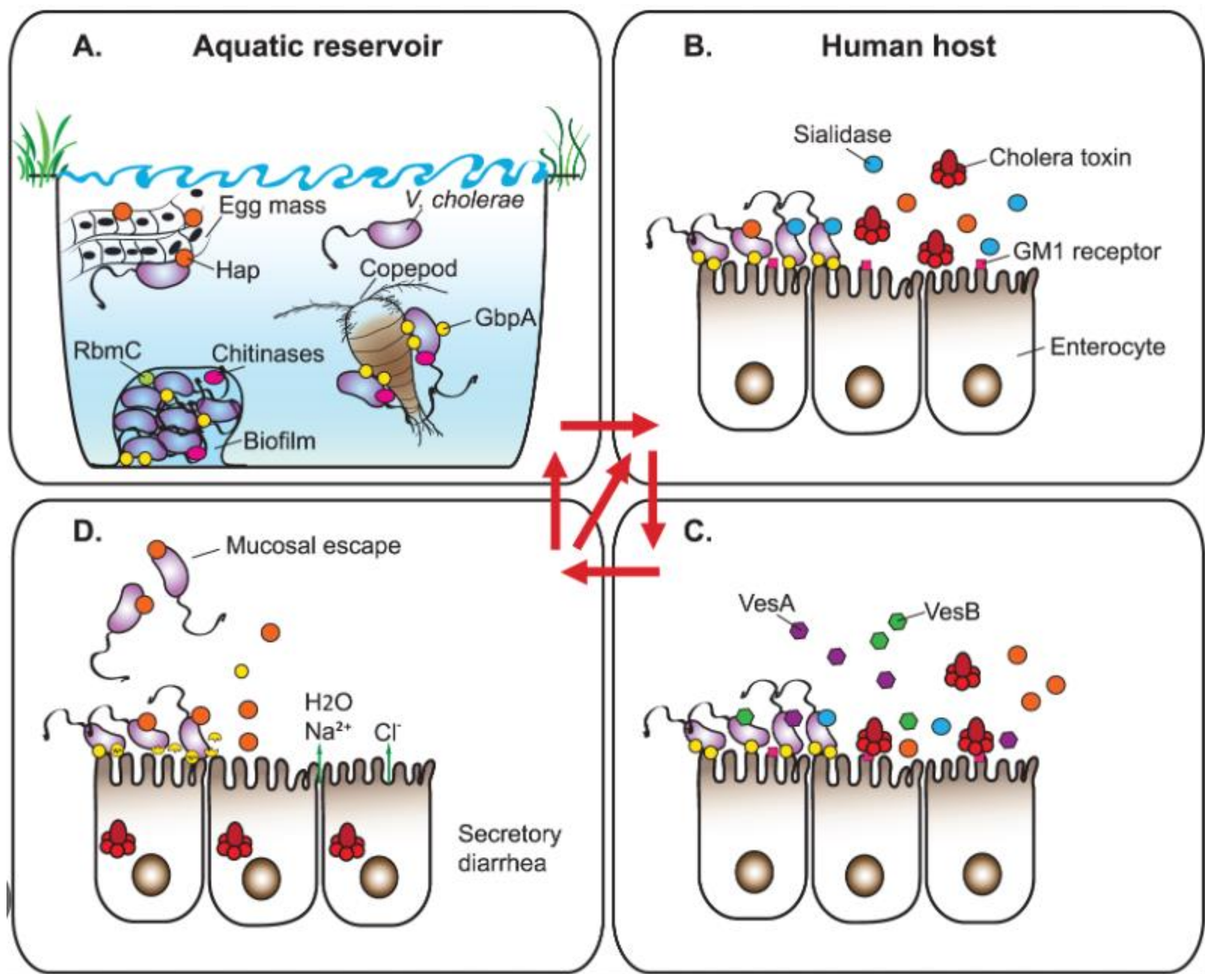

Figure 1.7. Illustration of the survival mechanisms of *V. cholerae*. The diagram A above depicts the aqueous, planktonic lifecycle and how it binds to various chitinous elements. B shows the binding and attachment of the bacterium to human epithelial cells via various glycan interactions. C follows the binding of cholera toxin to GM1 receptors. D depicts the disease effects of the toxin and the escape of *V. cholerae* back into the environment, thus continuing the cycle. Sikora, 2013.

Chapter 2

Research Design

2.1 Hypothesis of *Vibrio cholerae* adhesion

1. Mutated *V. cholerae* strains bind to different glycans when compared to wild type strains.
2. Truncated VPS, LPS and biofilm matrix protein strains (RbmA, RbmC, Bap1) of *V. cholerae* bind to fewer glycans when compared to the wild type strains.
3. VPS, LPS and biofilm matrix proteins are critically important in the aggregation of *V. cholerae*.
4. The growth and biofilm formation of mutant VPS, LPS and biofilm matrix protein strains (RbmA, RbmC, Bap1) of *V. cholerae* are less when compared to the wild type strains.

2.2 Aims of the project

1. To identify specific *V. cholerae* surface glycans involved in host-pathogen interactions.
2. To confirm the importance of VPS, LPS and the biofilm matrix proteins RbmA, RbmC and Bap1 in biofilm formation and their role in adhesion to Caco-2 cells.
3. To compare and contrast the effect of different monosaccharides on the biofilm of various *V. cholerae* strains.

Chapter 3

Materials

3.1 Chemicals and materials used in this study

Table 3.1: List of materials used in this thesis:

Media	***Constituents***
Luria-Bertani (LB) Broth (Oxoid)	10g/litre(l) tryptone[1], 5g/l yeast extract[1], 10g/l NaCl2 made up to 1 litre of distilled water.
Luria-Bertani (LB) agar	LB agar plates were prepared by adding 15g/l Agar Number 1[1].
PBS (Phosphate-buffered saline)	10 g of 25 g/L of LB powder, 400 mL of distilled water.
PBS Array	100 mL of distilled water, 20 mL of PBS-A, 20 mL of PBS-B, 300 mL of distilled water
Minimum Media (5X) solution	10 g of NH_4SO_4, 68 g of KH_2PO_4, 1 L of distilled water.
Magnesium (Mg) solution	49.29 g of MgSO4, 200 mL of distilled water.
Ethanol/Acetone solution	80 mL of Ethanol, 20 mL of Acetone (80:20, vol:vol)
Crystal Violet (0.1%)	0.1g / 100 mL of distilled water.
Caco-2 Cell culture media (DMEM) (ThermoFisher)	500mL DMEM, 50mL heat inactivated Foetal Bovine Serum (FBS), filtered through 0.45 nm and then 0.25 nm filters, 1% / 5mL of Penicillin Streptomycin Amphotericin (PSA).
Antibiotics (ThermoFisher)	Gentamycin (200 ug/mL)

3.2 *Vibrio cholerae* strains and growth conditions

3.2.1 Bacterial strains used in this study listed in table 3.1

Table 3.2: List of *V. cholerae* strains used in this study.

Name of strain	Genotype	Source:
Smooth rugose (Serogroup O1 El-Tor)	WT	Curtesy of Professor Fitnat Yildiz, University of California, Santa Cruz, USA
Rugose A1552 (Serogroup O1 El-Tor)	VPS overexpressing variant of smooth	Curtesy of Professor Fitnat Yildiz, University of California, Santa Cruz, USA
VPS 4327 (VPS lacking mutant of the rugose variant)	Δbap1	Curtesy of Professor Fitnat Yildiz, University of California, Santa Cruz, USA
LPS 13565 (LPS lacking mutant of the rugose variant)	Δgmb	Curtesy of Professor Fitnat Yildiz, University of California, Santa Cruz, USA

Biofilm matrix protein 4329 (mutant missing the following biofilm matrix proteins of the rugose variant)	ΔrbmA, ΔrbmC, Δbap1	Curtesy of Professor Fitnat Yildiz, University of California, Santa Cruz, USA
M1575 (Serogroup O1 El-Tor)	Epidemic and clinical isolates	Curtesy of Professor Routing Lan, UNSW, Sydney
M2559 (Serogroup O2)	Epidemic and clinical isolates	Curtesy of Professor Routing Lan, UNSW, Sydney
M552 (Serogroup O103)	Environmental isolates	Curtesy of Professor Routing Lan, UNSW, Sydney
M1566 (Serogroup O114)	Environmental isolates	Curtesy of Professor Routing Lan, UNSW, Sydney

Chapter 4

Methods

4.1 Glycan arrays

4.1.1 Cell growth

The *V. cholerae* strains were *V. cholerae* A1552 smooth (Serogroup O1 El-Tor), its rugose variant A1552 (Serogroup O1 El-Tor), biofilm matrix protein mutant 4329 (mutant missing the following biofilm matrix proteins of the rugose variant; ΔrbmAΔrbmCΔbap1), VPS mutant 4327 (VPS lacking mutant of the rugose variant), LPS mutant 13565 (LPS lacking mutant of the rugose variant).

V. cholerae cells were grown overnight on 2% LB Agar plates at 37°C. Cells were then harvested in LB broth solution. The cells were left on the bench in the presence of 1 mL of Formaldehyde (5% concentration) for 30 minutes at room temperature (24°C). The sample was then centrifuged at four thousand rpm at 4°C for 15 minutes to remove any remaining formaldehyde.

4.1.2 Fluorescent labelling of V. cholerae

One millilitre (1 mL) of PBS and 40 µL of Bodipy Dye (Fluorinated Boron-Dipyrromethene) were added to label the *V. cholerae* cells. Adequate labelling was ensured by shaking at 37°C at 100 rpm for 30 minutes. To remove the remaining dye, the sample was then centrifuged at 4000 rpm at 4°C for 30 minutes after which the supernatant was removed, 1 mL of PBS was added, and the solution was mixed thoroughly. This was repeated 3 times to ensure proper washing of the cells. After the final centrifugation, 20 mL of the buffer (PBS Array, Section 3.1) was added, and the OD was adjusted to 0.1 (Day et al. 2016).

4.1.3 Binding assays

Using gene frames, which consist of four sides of an adhesive material that can bind to glass and can contain liquid inside the square, two hundred micro litters (200 µL) of the cell sample were added onto a glycan slide. After the cells were incubated at room temperature for 30 minutes, protected from light in a dark room, the slides were gently washed with 1 mL of PBS Array to remove any excess dye and non-bind cells. The slides were then centrifuged at 4000 rmp for 5 minutes before being scanned in a Glycan Arrays Scanner at 480/520 wavelength. If the background was observed to be too high, the slides were washed again in PBS Array until a clear result was obtained (Day et al, 2016).

4.1.4 Image acquisition

The fluorescence intensity of array spots was measured using the ProScanArray Microarray 4-Laser Scanner (PerkinElmer) using the Blue Argon 480 excitation laser. The laser settings were set to the 480 excitation and 520 emission settings. Image analysis was carried out using the ProScanArray imaging software ScanArray Express (PerkinElmer).

4.1.5 Statistics

Microsoft Excel was used to further process the data and to perform statistical analysis by an independent sample T test (Day et al., 2016).

4.2 Biofilm assays

4.2.1 Cell growth

V. cholerae cells were grown overnight on 2% Luria-Bertani (LB) agar plates in 37°C. Cells were harvested in Minimum Media (1XMg) solution. The OD_{600} was measured using the Infinite M200 Pro (Tecan) plate reader and adjusted to 0.1. Cells were transferred to a 24 Multi-well (Falcon) culture plate and monosaccharides were added. *V. cholerae* cells were allowed to grow overnight at 37°C.

4.1.3 Biofilm quantification

The biomass of *V. cholerae* biofilms was quantified by a Crystal Violet (CV) staining assay (Ryuto et al., 2022). Briefly, the supernatant was removed, and 400 mL of 0.1% CV solution was added to the wells. After a 10 min incubation time, the CV was removed, and each well was gently washed with water three times. To dissolve or suspend the CV from the bottom of the wells, 400 uL of Ethanol/Acetone solution was added. The spectrophotometer (plate reader Infinite M200 Pro (Tecan)) was then used to measure and record the OD results of each well at 590 nm.

4.3 Auto-aggregation assays

4.3.1 Cell growth

V. cholerae cells were grown overnight on 2% LB Agar plates at 37°C. Cells were harvested in Minimum Media (1XMg) solution, which is clear for a good visual result. To ensure each strain had the same number of cells, the OD was adjusted to 0.5.

4.3.2 Cell aggregation

After the cells were transferred to 50 mL tubes, the appropriate monosaccharides of glucose, galactose, mannose, glucosamine, fucose, and in addition, mucin, were added. To observe a clear aggregation, the cells were left for 24 hours on a still surface, as to not disturb the aggregation. The OD of each supernatant of each strain was then measured and photos were taken as a visual representation of the OD results.

4.4 Adhesion and invasion assays

4.4.1 Culture of caco-2 cells

Caco-2 cells (1×10^5 cells/well, American Type Culture Collection) were grown at 37°C in minimal essential medium (MEM, Gibco Laboratories) supplemented with 10% (vol/vol)

foetal bovine serum (FBS) in a humidified atmosphere containing 5% CO2 and 95% air. Confluent cells were harvested by trypsinization in 0.01% EDTA (BDH) and plated in a 12-well Multiwell (Falcon) tissue culture plate. Cells were fed with fresh media every two days. Polarized Caco-2 cell monolayers were used after 5 days of growth (Szymanski, 1995).

4.4.2 Adherence assay to cultured caco-2 cells

VC cells were co-cultured with human Caco-2 cells, and the adherence rate was measured as previously described (Day et al, 2016). Adherence rate was calculated against the size of the inoculum, (~10^8 bacterial cells per human cell in confluent cell culture). The percentage of internalization was calculated based on the following formula: total count of internalized pathogenic bacteria/ total number of inoculated pathogenic bacteria (10^8 CFU)×100 (Alamdary, 2018).

4.4.3 Invasion assay to cultured caco-2 cells

VC cells were co-cultured with human Caco-2 cells, and the adherence rate was measured as previously described (Day et al, 2016). Adherence rate was calculated against the size of the inoculum, (~10^8 bacterial cells per human cell in confluent cell culture). The percentage of internalization was calculated based on the following formula: total count of internalized pathogenic bacteria/ total number of inoculated pathogenic bacteria (10^8 CFU)×100 (Alamdary, 2018).

After the 4 hours of incubation, the media removed by aspiration and replaced with fresh media containing 200 ug/ml gentamicin and re-incubated for 4 hours at 37°C in the CO_2 incubator.

4.4.4 Viability assay

A viability assay was conducted to confirm the effectiveness of gentamicin. *V. cholerae* cells grown overnight at 37°C on LB plates were harvested and transferred into a 24 Multi-well (Falcon) culture plate. All strains were tested against 100 ug/mL and 200mg/mL of gentamicin over 24 hours at 37°C.

Chapter 5

Results

5.1. Assessment of biofilm formation capability of environmental and clinical *Vibrio cholerae* strains

To compare the biofilm formation of clinical and environmental strains, a biofilm assay was performed for strains wild type (smooth) A1552, biofilm overproducing rugose variant of A1552, M552 (O103) (environmental isolate), M1575 (O1 El-Tor) (clinical isolate), M1566 (O114) (environmental isolate) and M2559 (O2) (clinical isolate) (Section 4.3.1).

In this study, we investigated whether there was an increase or decrease in the biofilm formation by various *V. cholerae* strains by leaving cells in minimum media in 24 well plates. After 24 hours, the contents of the wells were stained with CV and washed. The OD_{590} of the formed biofilm was then measured for all samples (Figure 5.1).

As shown by Figure 5.1, a significant difference ($p \leq 0.0001$) was detected in the biofilm formation by the A1552 rugose (O1 El-Tor) variant when compared to the A1552 smooth (O1 El-Tor) wild type strain. Also, the environmental strains M552 and M1575, as well as the clinical strains M1566 and M2559, naturally produced more biofilm when compared to the A1552 smooth wild type strain ($p \leq 0.0001$).

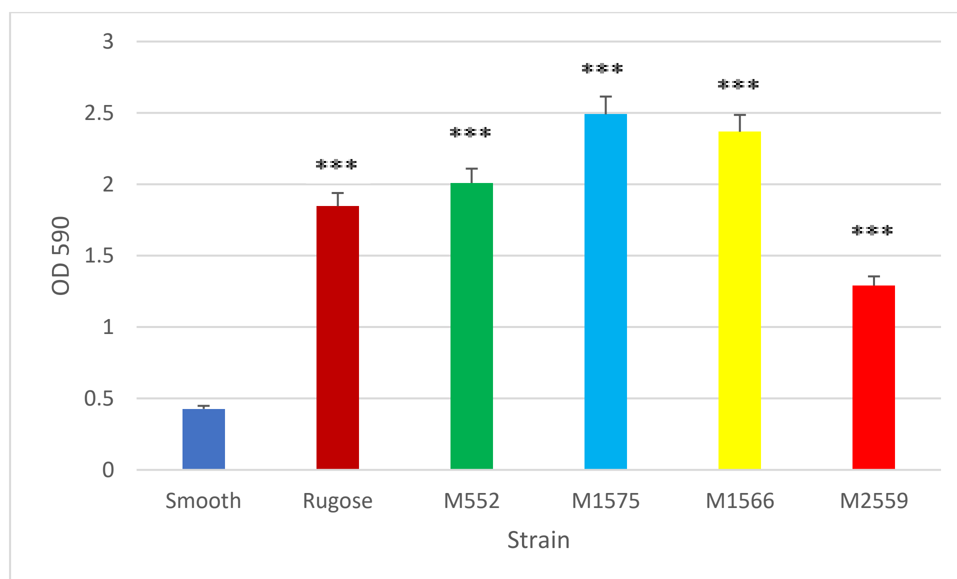

Figure 5.1. Biofilm production by *V. cholerae* strains. Each bar represents the mean of three replicates; standard error is indicated by error bars. *** equates to a $p \leq 0.05$ compared to A1552 smooth.

Unexpectedly, it was observed that both the environmental and clinical strains produced more biofilm when compared to the A1552 smooth wild type strain.

5.1.1 Adhesion of environmental and clinical Vibrio cholerae strains to caco-2 cells comparing to A1552 smooth wild type and its rugose variant

To compare the adhesion of clinical and environmental strains, an adherence assay using Caco-2 cells was performed for different strains, the wild type *V. cholerae* strain (smooth A1552, its rugose variant A1552, M552 (O103) (environmental isolate), M1575 (O1 El-Tor) (clinical isolate), M1566 (O114) (environmental isolate) and M2559 (O2) (clinical isolate) (Section 4.3.1).

In this study, we investigated whether there was a natural difference in the biofilm of various *V. cholerae* strains by leaving *V. cholerae* cells in the presence a Caco-2 monolayer in DMEM media over a period of four hours. The percentage of adherence was then calculated for the wild type, environmental and clinical strains.

Figure 5.1.1 shows that, the VPS overproducing rugose variant of A1552 had a greater adherence to Caco-2 cells when compared to the A1552 smooth wild type strain ($p \leq 0.0001$). The environmental strain 552 ($p \leq 0.01$) and the clinical strain 1575 ($p \leq 0.0001$) had less adherence to Caco-2. The environmental strain 1566 ($p \leq 0.0001$) and the clinical strain 2559 ($p \leq 0.0001$) naturally had more adherence when compared to the A1552 smooth wild type strain.

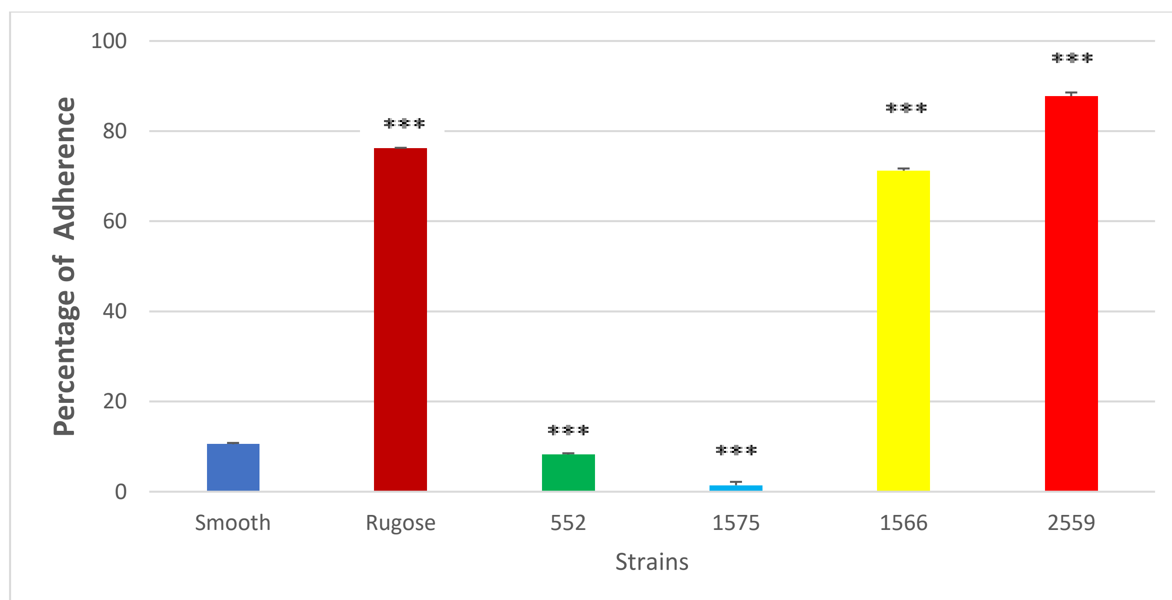

Figure 5.1.1: *V. cholerae* adherence to Caco-2 cells. Each value indicates the mean ± SEM from two independent experiments and three replicates (n = 3). * Indicates $p \leq 0.05$ as a significantly difference.

The environmental strain 552 and clinical strain 1575 strain had little adhesion to Caco-2 cells, with similar levels to the A1552 wild type. The other environmental strain 1566 and clinical strain, 2559, both had higher levels of adhesion to Caco-2 cells, with similar adhesion to the rugose variant of A1552. Thus, no distinction can be made between environmental and clinical strains until more strains are investigated.

5.1.2 Analysis and discussion

All four environmental and clinical strains had naturally formed more biofilm when compared to the A1552 wild type strain. However, since the biofilm levels between these four environmental and clinical strains were quite similar, more study is needed before any distinction can be made. As biofilm formation is crucial for survival of this bacteria in aquatic environments, it is expected for clinical strains would produces more biofilm. This is supported by the literature (Lizárraga-Partida, 2009). Why these strains produce less biofilm, however, is still unknown.

Unexpectantly, 1 clinical (1575) and 1 environmental (552) strain showed similar adhesion levels when compared with the A1552 smooth wild type strain, while the other clinical (2559) and environmental (1566) strain showed adhesion similar when compared with the VPS overexpressing rugose variant of A1552. However, more study is needed into these strains to determine what mediates their adhesion to Caco-2 cells.

5.2 Analysis of glycan binding using the glycan arrays

V. cholerae has multiple complex interactions that bring it into contact with many different molecules and glycans in its lifespan. Previous studies have shown that Cholera Toxin interacts with human ganglioside GM1 (Merritt et al., 1994; De et al., 2018), while the biofilm matrix protein RbmA can bind galactose, fucose and sialic acid (De et al., 2018). Few other potential interactions with the host molecules, such as blood group antigens, had been suggested based on epidemiological and population studies (El-Hawiet et al., 2015; Lambert & Neuhaus, 1972; Tribble et al., 2010). VPS and LPS have both shown to contain glucose and galactose within their structures, with VPS further containing mannose and N-acetyl-glucosamine groups (Fong, 2010). With VPS, LPS and biofilm matrix proteins playing critical roles in the adhesion and biofilm formation of *V. cholera*, a full range of glycans binding to these truncated stains would be insightful into the glycan-glycan host bacterial interactions.

In this study, we investigated the ability of *V. cholerae* to bind mono, di, tri and complex glycans. Bacterial cells were fluorescently labelled and bound to a printed Glycan Array glass slide, which contained four hundred glycans. The binding spots were then washed and scanned to determine which glycans bound to the selected strains. The results were then visualized as heat maps.

In order to test how the absence of functional VPS would affect *V. cholerae's* ability to bind to glycans, the VPS mutant strain 4327 was used. The LPS mutant strain 13565 was chosen to test the effect of truncated LPS, while the biofilm matrix protein mutant strain 4329 was included since it didn't have the biofilm matrix proteins present. All 3 strains are derived from the rugose variant of the A1552 smooth wild type strain. Thus, these strains were tested to identify any differences in glycan binding between strains and to compare this to the wild type and its rugose variant.

5.2.1 Glycan interactions: matrix

V. cholerae strain A1552 smooth wild type and its rugose variant were found to bind simple and complex glycans, while the biofilm matrix protein mutant strain 4329 was found to not bind to simple and complex glycans.

The results shown in Figure 5.2.1 are a representative selection of all the glycan-cell interactions, which can be found in the Supplementary data (Table S1 and S2). Each result is an average of the 3 biological repeats.

V. cholerae strain A1552 smooth wild type and its rugose variant, were able to interact with monosaccharides (glucose, galactose, mannose and fucose), disaccharides (sucrose, lactose, maltose) and polysaccharides including fucosylated glycans, sialylated glycans, terminal galactose, terminal glucosamino, and mannosyl-linked complex glycans (Figure 5.2.1). However, the biofilm matrix protein mutant strain 4329, has lost the ability to interact with most of the glycans. Both *V. cholerae* strain A1552 smooth wild type and its rugose variant also bound human cell surface associated glycans such as gangliosides (i.e., GM1), blood group antigens and Lewis antigens (i.e., LeY), while the biofilm matrix protein mutant strain 4329 did not. Interestingly, only the rugose variant could bind to blood group H and P antigens, indicating that overproduction of VPS may allow additional interactions with the host cell.

We determined that *V. cholerae* strain A1552 smooth wild type and its rugose variant could bind to human gangliosides, Lewis (Le) and other blood group antigens present on human host cells. Specifically, we found that *V. cholerae* strain A1552 smooth wild type and its rugose variant cells were able to bind blood group A, B, LacNAc, Lea, Leb, Lex, Ley as well as the core Lec antigens, all of which are abundantly expressed in the intestinal epithelial cells. Terminal human ganglioside structures bound by *V. cholerae* strain A1552 smooth wild type and its rugose variant included GM1, asyalo GM1, GM2 and GD. Other cell surface antigens were also recognised, such as laminin, vimentin, Thomson-Friedeneich and TFLN, the last usually found in human milk. Interestingly, H blood group antigen, characteristic of blood group O, was not bound by either *V. cholerae* strain A1552 smooth wild type or its rugose variant. A wide range of mono-, di-, tri- and tetrasuccharides with terminal or branched fucose, galactose, GalNAc, GlcNAc, and Neu5Ac (sialic acid), abundant on cell-surface (MUC1) , secreted (MUC2) mucins, mannose , terminal or branched , all bound both *V. cholerae* strain A1552 smooth wild type and its rugose variant.

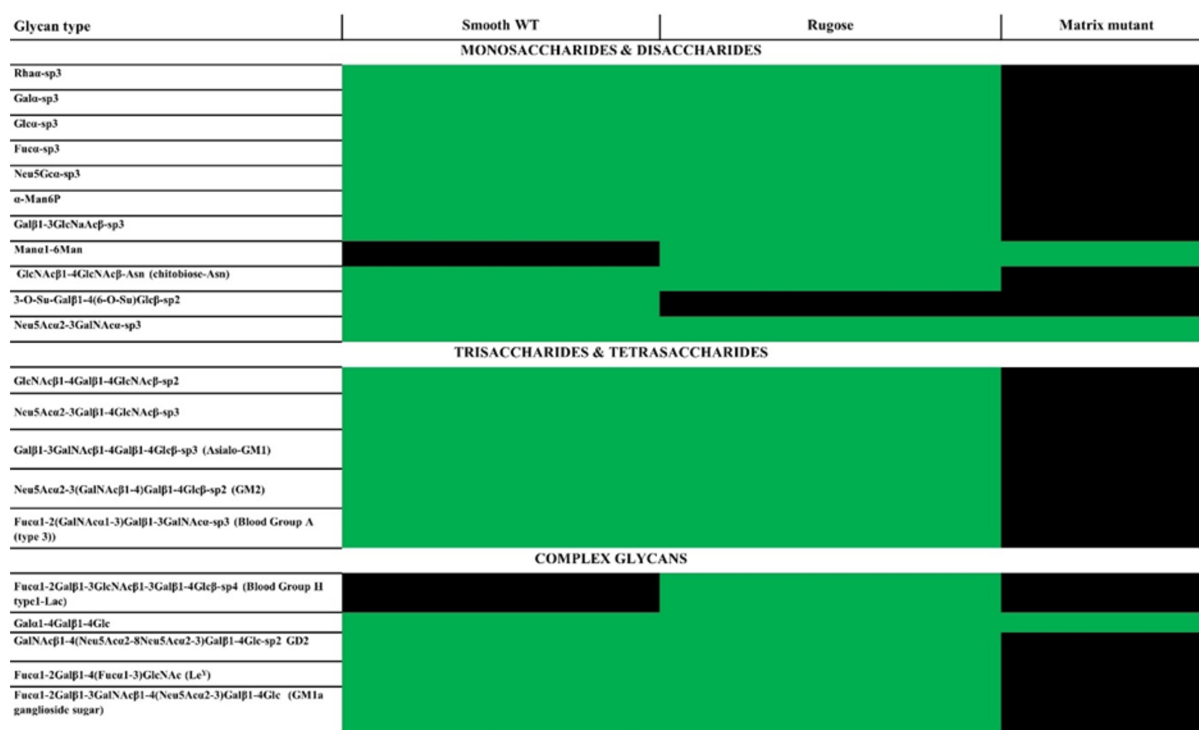

Figure 5.2.1: Heat map of structures seen by Glycan arrays. An example of the whole- cells *V. cholerae* interactions with glycan arrays. The figure above is a heat map of some of the glycan structures recognized by A1552 smooth wild type, its rugose variant and the biofilm matrix protein mutant 4329.

5.2.2 Glycan interactions: VPS and LPS

We have then compared the differences in the ability to bind glycans between *V. cholerae* strain A1552 smooth wild type and its rugose variant to the VPS mutant 4327 and LPS mutant 13565.

The VPS mutant 4327 and LPS mutant 13565 were found to not bind simple and complex glucans and did not bind to human cell surface associated glycans (i.e., GM1), blood group antigens and Lewis antigens (i.e., Le^{Y}), as compared to *V. cholerae* strain A1552 smooth wild type and its rugose variant. Interestingly, while the rugose variant and the VPS mutant 4327 both showed the ability to bind to blood group H and P antigens, the LPS mutant 13565 has lost that binding. We also determined that the rugose variant, the VPS mutant 4327 and LPS mutant 13565 binds to Manal-6Man, while *V. cholerae* strain A1552 smooth wild type does not.

Interestingly, the LPS mutant 13565 and the VPS mutant 4327 have both lost all binding for this set of trisaccharides and tetrasaccherides, indicating the importance of LPS and VPS in binding these sugars (Figure 5.2.2).

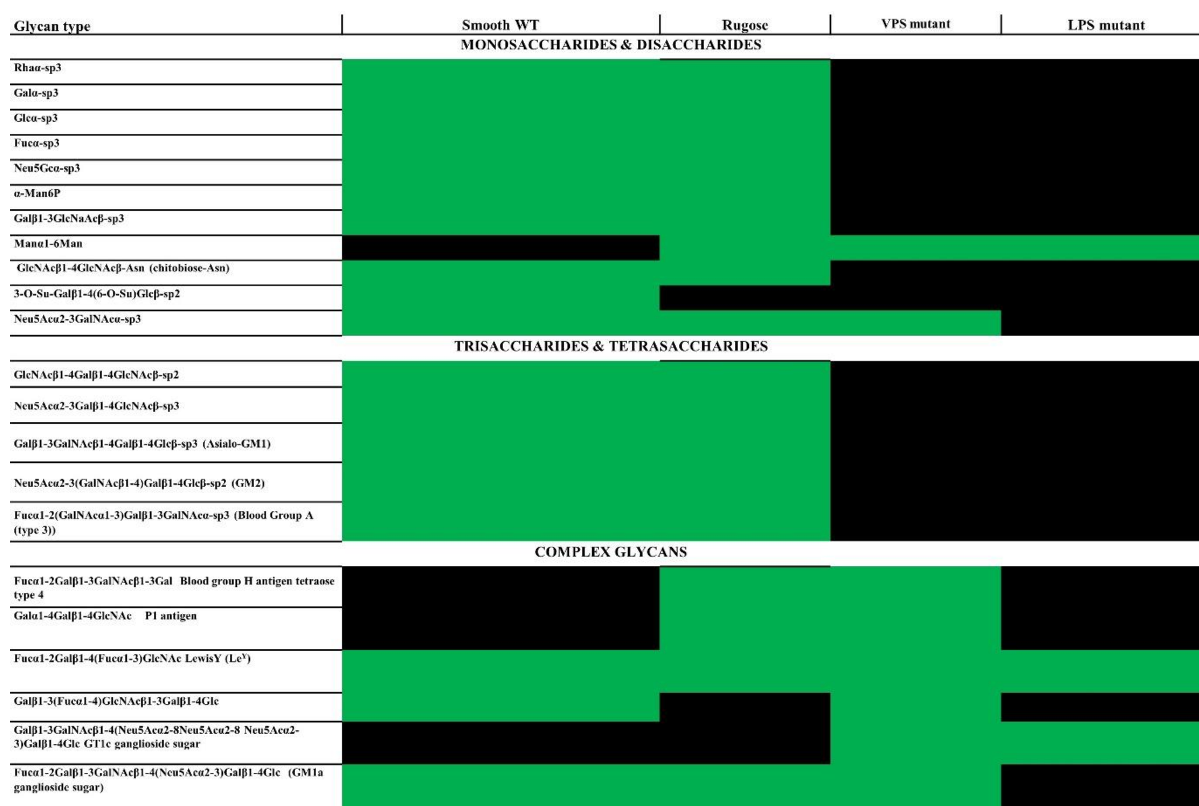

Figure 5.2.2: Heat map of structures seen by Glycan arrays. An example of the whole- cell *V. cholerae* interactions with glycan arrays. Heat map of glycan structures recognized by A1552 smooth wild type, its rugose variant, the VPS mutant 4327 and the LPS mutant 13565.

These findings suggest that the VPS mutant 4327, the LPS mutant 13565 and the biofilm matrix protein mutant 4329 have all lost the ability to bind to most of the glycans. This may indicate that VPS, LPS and biofilm matrix proteins are all important in binding *V. cholerae* to these glycans.

5.2.3 Analysis and discussion

It has long been established that the recognition of glycan receptors on host cell-surfaces by *V. cholerae* is an important strategy for achieving the selectivity and potency of virulence factors including adhesion molecules, toxins, and biofilm proteins (Kato & Ishiwa, 2015; Silva & Benitez, 2016; De et al., 2018).

We confirmed the binding of human gangliosides (GM1), Lewis (Le) and other blood group antigens present on human host cells to both wild type El Tor strain A1552 smooth wild type and its rugose variant. These human cell surface antigens have previously been associated with the cholera outbreaks via statistical association and population analysis (Harris et al., 2005). Furthermore, both the A1552 smooth wild type strain and its VPS overexpressing rugose variant bound to blood group A, B, LacNAc, Lea, Leb, Lex, Ley as well as the core Lec antigens, all of which are abundantly expressed in the intestinal epithelial cells. Thus, binding to these antigens may lead to successful colonisation of *V. cholerae* in the gut (Breimer et al., 2012).

Interestingly, H blood group antigen, a characteristic of blood group O, was not bound by either strain, in agreement with population data that people with this blood group may be less susceptible to initial infection. It was also shown, however, that once a patient was infected, having blood group O was associated with an increased risk of severe cholera. Therefore, further tests into the importance of the H blood group antigen are needed.

We confirmed that the rugose variant bound to mannose, either terminal or branched. Mannose plays an important role in adhesion and colonisation of the rugose variant strain for biofilm formation (Watnick et al., 1999); thus, an overexpression of VPS may increase binding to mannose and thus aid in colonisation.

The A1552 smooth wild type strain and its VPS overexpressing rugose variant were able to bind to N-glycolylneuraminic acid (Neu5GC) which is a sialic acid molecule found in most non-human mammals, skin of fish and surface of shellfish. It is not, however, found in human tissues. In contrast, N-acetylneuraminic acid, Neu5Ac, which was also bound by *V. cholerae*, is considered to be an evolutionary human adaptation (Alisson-Silva et al., 2018). This finding was consistent with the dual lifestyle of *V. cholerae*, as was the binding to chitin polymers, which are associated with attachment and biofilm formation on the surface of shellfish and other marine inhabitants. We confirm that the rugose variant is able to bind to chitin-derived oligosaccharide, chitobiose and chitotriose, while the A1552 smooth wild type strain is not. Again, this may be due to the over-expression of VPS, which may cause an increase in binding and biofilm formation. Further research, however, is needed.

This study also showed that the biofilm matrix protein mutant 4329 did not bind to a wide range of mono, di, tri and tetra saccharides, indicating that the biofilm matrix proteins play a critical role in the binding of *V. cholerae* to these sugars.

We report that the biofilm matrix protein mutant 4329 did not bind to a wide range of simple and complex sugars, such as galactose, fucose, sialic acid, rhamnose, and N-acetyl galactosamine. Here we show that the biofilm matrix protein RbmA did not bind to these saccharides, and thus had severe adhesion and colonisation deficiencies, as supported by (Teschler, 2015).

In addition, the biofilm matrix protein mutant 4329 did not bind to a wide range of complex glycans, such as GM1, Blood group H and P antigens and Lewy body Le^{Y}, indicating that the biofilm matrix proteins play a critical role in the binding of *V. cholerae* to these complex glycans. Further study may be needed to determine the exact effects of each of the biofilm matrix proteins.

The VPS mutant strain 4327 did not bind to most glycans, such as fucose, mannose, galactose, GalNAc- 2′-N-acetyl galactosamine, glucose, GlcNAc- 2′-N-acetyl glucosamine. This is consistent with the literature (Fong, 2010). However, the A1552 smooth wild type strain and its rugose variant, as well as the VPS mutant 4327, did surprisingly bind to Neu5Acα2. This glycan is usually involved with influenza and mucus production (Wang, 2018). Thus, we hypothesise that VPS is not important in binding *V. cholerae* to this glycan, however, further studies are needed to confirm this.

Surprisingly, we can see that the rugose variant of A1552, the VPS mutant 4327 and the LPS mutant 13565 bound to Manal-6Man, while the A1552 smooth wild type strain did not (Figure 5.2.2). This was an unexpected result as the A1552 smooth wild type strain did not bind to it, but its truncated mutants did. However, this requires further investigation before any conclusion can be made.

Interestingly, while the rugose variant of A1552 and the VPS mutant 4327 both showed the ability to bind to blood group H and P antigens, the LPS mutant 13565 has lost that binding ability (Figure 5.2.2). This may indicate the importance of LPS in binding to blood groups and people's susceptibility to *V. cholerae* based on blood group. This warrants further study and research.

We confirm that the LPS mutant 13565 did not bind to fucose, mannose, galactose, GalNAc- 2'-N-acetyl galactosamine, glucose, GlcNAc- 2'-N-acetyl glucosamine. Surprisingly, the LPS mutant 13565 does bind to Le^{Y} bodies, indicating that LPS may not be important in the binding of Lewy bodies.

This study showed that the LPS mutant 13565 did not bind to a wide range of mono, di, tri and tetra saccharides, indicating that LPS plays has a critical involvement in the binding of *V. cholerae* to these sugars.

5.3 Analysis of auto-aggregation by *Vibrio cholerae* strains and their mutants

It has been shown that biofilm is critical in the pathogenicity and virulence of *V. cholerae* (Thomas, 2018; Ramamurthy et al., 2020). Thus, the formation of the biofilm is both important and necessary for colony establishment and disease progression of *V. cholerae* (Chiang, 1995; Garcia-Peñarrubia et al., 2002). Auto-aggregation is the first step in a series of events that lead to the formation of biofilms and eventual pathogenesis. Auto-aggregation occurs when *V. cholerae* cells detect self-recognizing surface structures and clump together. These so-called auto-agglutinins include proteins and exopolysaccharides, and mediate aggregation between the same bacteria (Thomas, 2018).

Glycans are involved in the production of both biofilm formation and the binding and adhesion of *V. cholerae* to host cells. Therefore, since so many different glycans bind to the *V. cholerae* strains, such as the Cholera Toxin binding to GM1 gangliosides (Crevin et al., 2018), it was decided to add these monosaccharides to *V. cholerae* strains A1552 smooth wild type, its rugose variant, the VPS mutant 4327, the LPS mutant 13565 and the biofilm matrix protein mutant 4329 and observe if there is a significant increase or decrease on the auto-aggregation of these strains.

Here we investigated the auto-aggregation ability of various *V. cholerae* strains in the presence of 50 mM of galactose over a 24 hour period and compared that to the natural auto-aggregation ability of various *V. cholerae* strains.

Galactose was chosen as it is a key glycan that *V. cholerae* strains bind to, and previous studies have suggested that it may have an inhibitory effect on auto-aggregation. Thus, we tested whether galactose might interfere with the auto-aggregation of *V. cholerae* strains.

5.3.1 Visual assessment of auto-aggregation capability of Vibrio cholerae A1552 rugose variant and the 13565 LPS mutant strain

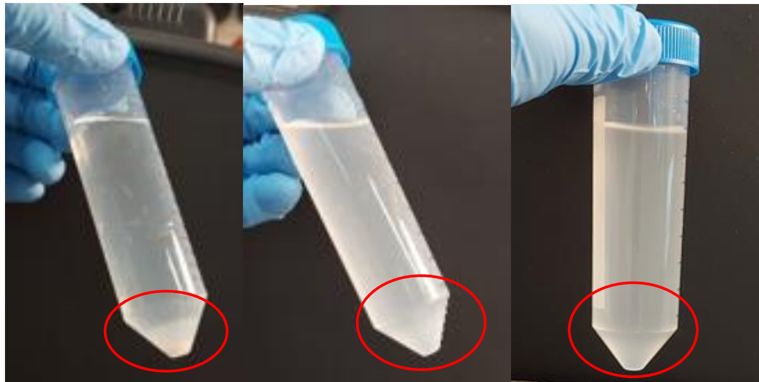

Figure 5.3.1: Macroscopic visualisation of *V. cholerae* cells. Above are images of the *V. cholerae* LPS mutant 13565 strain without 50 mM of galactose (left), the *V. cholerae* LPS mutant 13565 strain with the presence of 50 mM of galactose (middle) and the A1552 wild type strain without 50 mM of galactose (right).

As seen in Figure 5.3.1 above, there is a large and distinct visual difference between the aggregation of the LPS mutant 13565 cells without 50mM of galactose and the aggregation of the LPS mutant 13565 cells with 50mM of galactose . The solution is clear in the image on the left, with a very large pellet formed at the bottom of the tube. When 50 mL of galactose was added to the LPS mutant 13565 cells, the solution remained turbid, and appeared similar to the A1552 wild type phenotype.

5.3.2 Quantitative assessment of auto-aggregation capability of Vibrio cholerae strains

To further understand the involvement that VPS, LPS and biofilm matrix proteins may play in auto-aggregation, an auto-aggregation assay was performed with the mutant strains (A1552 smooth wild type, its rugose variant, the VPS mutant 4327, the LPS mutant 13565, the biofilm matrix protein mutant 4329) (Section 4.3.2). The OD was measured at time zero at OD 0.5 to act as a control and to ensure we had the same number of cells in all samples. The OD of the planktonic cell suspension was measured after 24 hours for all samples not containing 50 mM of galactose and for all samples containing 50 mM of galactose (Figure 5.3.2).

Figure 5.3.2 shows that a significant decrease of 14% in the quantity of cells of the rugose variant remained in suspension as compared to that of A1552 smooth wild type ($p \leq 0.05$). A significant decrease in the cells was detected for the LPS mutant 13565 strain when compared to the rugose variant ($p \leq 0.001$) and to A1552 smooth wild type ($p \leq 0.0001$). No significant decrease of cells remaining in suspension was detected for the VPS mutant 4327 ($p \geq 0.1$) and biofilm matrix protein mutant 4329 ($p \geq 0.1$) strains when compared to the rugose variant. No significant decrease of in suspension cells was detected for the VPS mutant 4327 ($p \geq 0.5$) and biofilm matrix protein mutant 4329 ($p \geq 0.1$) strains when compared to A1552 smooth wild type strain.

Furthermore, Figure 5.3.2 shows that when 50 mM of galactose is added to the LPS mutant 13565 cell suspension, a significant decrease in the auto-aggregation could be observed when compared to the rugose variant strain with 50 mM of galactose ($p \leq 0.005$) and the A1552 smooth wild type strain with 50 mM of galactose ($p \leq 0.0001$). No significant decrease in the auto-aggregation was detected in the VPS mutant 4327 ($p \geq 0.1$) and biofilm matrix protein mutant 4329 ($p \geq 0.1$) strains with 50 mM of galactose when compared to the rugose variant with 50 mM of galactose. No significant decrease in the auto-aggregation was detected in the VPS mutant 4327 ($p \geq 0.1$) and biofilm matrix protein mutant4329 ($p \geq 0.1$) strains with 50 mM of galactose when compared to A1552 smooth wild type with 50 mM of galactose.

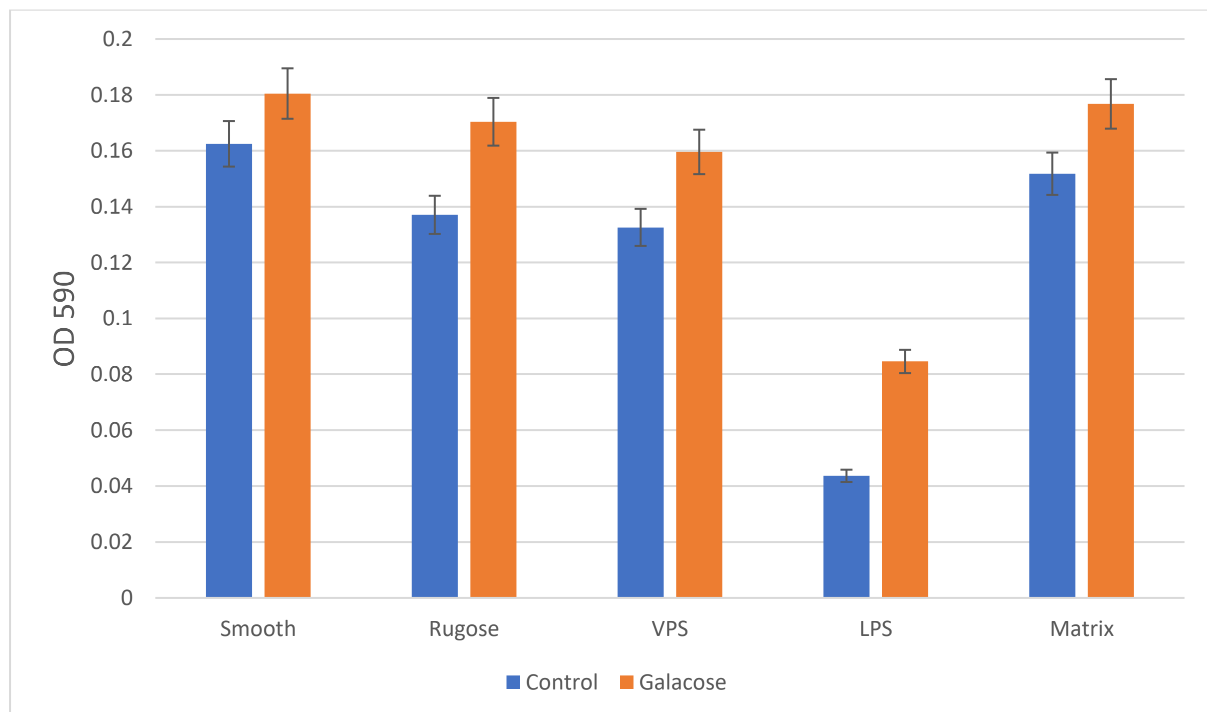

Figure 5.3.2. OD measurements of *V. cholerae*. OD measurement of cells remaining in suspension following auto-aggregation of various *V. cholerae* strains with and without 50 mM of galactose. Each bar represents the mean of three replicates; standard error is indicated by error bars. *** equates to $p \leq 0.05$ compared to A1552 smooth wild type and/or its rugose variant.

5.3.3 Analysis and discussion of auto-aggregation results

The auto-aggregation assay found that the rugose strain had a significant increase in auto-aggregation (and a corresponding lower planktonic reading) when compared to the wild type A1552 smooth wild type strain (Figure 5.3.2, $p \leq 0.05$), indicating that VPS is especially important in creating and binding the biofilm (Rashid et al., 2003, Chowdhury et al., 2016). Since the A1552 rugose variant strain is an overexpressing variant of A1552 smooth wild type, we can see that an abundance of VPS has a significant effect on the auto-aggregation of *V. cholerae*.

The VPS mutant 4327 and biofilm matrix protein mutant 4329 were found to have no significant difference in auto-aggregation when compared to the rugose variant or the A1552 smooth wild type. It appears that the VPS and biofilm matrix protein mutations did not affect auto-aggregation. Thus, we speculate that the involvement of VPS and the biofilm matrix proteins may be limited in auto-aggregation.

There was a significant increase in quantity of suspended cells of the LPS mutant 13565 when galactose was added (Figure 5.3.2, $p \leq 0.001$). This corresponds to a significant decrease in the auto-aggregation of the LPS mutant. During auto-aggregation, cells adhere to each other and precipitate (Teschler, 2015). When galactose was added, there may have been a reduction in the adhesion between cells, thus causing less auto-aggregation. Possible future research avenues may include competitive inhibition assays (Rehmat et al., 2019) or NMR spectroscopy (Monaco, et al., 2020).

Thus, it is suggested that glycans, especially galactose, may have the ability to inhibit the biofilm formation of *V. cholerae*, especially the LPS mutant 13565 strain. It was shown in the glycan array results that WT *V. cholerae* binds to many glycans, while its VPS mutant 4327, LPS mutant 13565 and biofilm matrix protein mutant 4329 do not bind to most glycans. We have shown that the VPS, LPS and biofilm matrix protein mutants have reduced auto-aggregation, and since it is known that auto-aggregation is part of the biofilm formation process, it was decided to test the effects of glycans on the biofilm itself of *V. cholerae*.

5.4 Analysis of biofilm formation by *Vibrio cholerae* strains A1552 smooth wild type, its rugose variant, and their VPS, LPS and biofilm matrix protein mutants

V. cholerae actively forms and maintains biofilms, surface-attached communities of bacterial cells which are embedded in a self-produced polymeric matrix (Silva & Benitez, 2016; Wolska, 2016; Thomas, 2018). *V. cholerae* cells attached to target surfaces, which leads to reversible and irreversible binding, culminating in microcolony formation and mature biofilm formation (Yildiz, F. H., & Visick, K. L., 2009; Rodney , 2001; Thomas, 2018).

These biofilms are comprised of a number of different components, such as important polysaccharides, such as VPS (Staropoli & Alon, 2000., Fong , 2010) and LPS (Iredell et al.,

1998; Mohammad Pour Ghazi & Gargari, 2016), and critical proteins such as biofilm matrix proteins (Fong , 2007; Teschler, 2015, Wolska, 2016). The biofilm also contains glucose and galactose as part of the structure of VPS (Wolska, 2016).

VPS has been shown to be of critical importance in the formation of 3-dimensional biofilm structures (Teschler, 2015, Wolska, 2016), while LPS has a role to play in adhesion and protection (Nesper, 2001). VPS also works together with biofilm matrix proteins to incorporate VPS into the biofilm structure and provides elasticity and stability to the forming biofilm (Teschler, 2015).

Since the glycan arrays showed that the VPS, LPS and biofilm matrix protein mutants did not bind to most glycans, it was decided to evaluate the potentially inhibitory effects of saccharides on the biofilm.

Therefore, we investigated if there was an increase or decrease of the biofilm of various *V. cholerae* strains by leaving cells in 24 well plates in the presence of monosaccharides.

5.4.1 The involvement of VPS, LPS and biofilm matrix proteins in biofilm formation

To understand the involvement of VPS, LPS and biofilm matrix proteins in biofilm formation, a biofilm assay was performed with the wild type A1552 smooth wild type, its rugose variant and its VPS (4327), LPS (13565), and biofilm matrix protein (4329) mutant strains. Each strain was grown with or without the glycans, and the biofilm formation was measured after 24 hours. All saccharides were adjusted to 50 millimolar (mM). The results are displayed in Figure 5.4.1a – 5.4.1f and detailed analysis in the following sections.

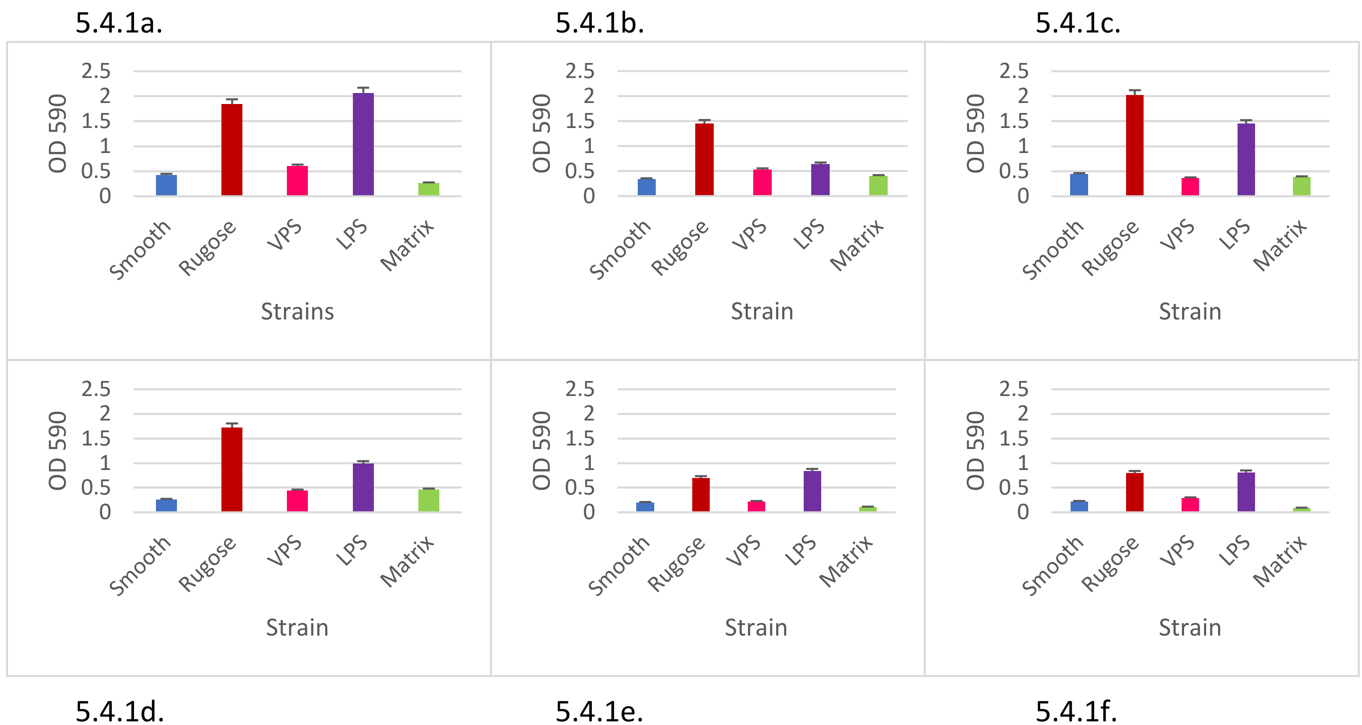

Figure 5.4.1a – f. Biofilm production by *V. cholerae* strains with and without additives. Each bar represents the mean of three replicates; standard error is indicated by error bars. From left to right, top to bottom, 5.4.1a. *V. cholerae* strains and mutants without additives. 5.4.1b. *V. cholerae* strains and mutants in the presence of 50 mM of galactose. 5.4.1c. *V. cholerae* strains and mutants in the presence of 50 mM of mannose. 5.4.1d. *V. cholerae* strains and mutants in the presence of 50 mM of glucosamine. 5.4.1e. *V. cholerae* strains and mutants in the presence of 50 mM of fucose. 5.4.1f. *V. cholerae* strains and mutants in the presence of 50 mM of mucin.

5.4.2 Effects of galactose, mannose, glucosamine, fucose and mucin on Vibrio cholerae strain A1552 wild type biofilm formation

Galactose, mannose, glucosamine, fucose and mucin (as control) were added to *V. cholerae* strain A1552 smooth wild type to determine if these monosaccharides were able to inhibit biofilm formation.

Figure 5.4.2 show that there was no significant difference detected in the amount of biofilm formed by the wild type strain when mannose ($p \leq 0.6499$) and galactose ($p \leq 0.1216$) were added, as compared to un-supplemented cells. However, when glucosamine ($p \leq 0.0173$), fucose ($p \leq 0.0057$) and mucin ($p \leq 0.0080$) were added, there was a significant decrease in the amount of biofilm formed. This suggests that specific sugars, such as glucosamine and fucose, and mucin may have an inhibitory effect on the *V. cholerae* strain A1552 smooth wild type biofilm formation.

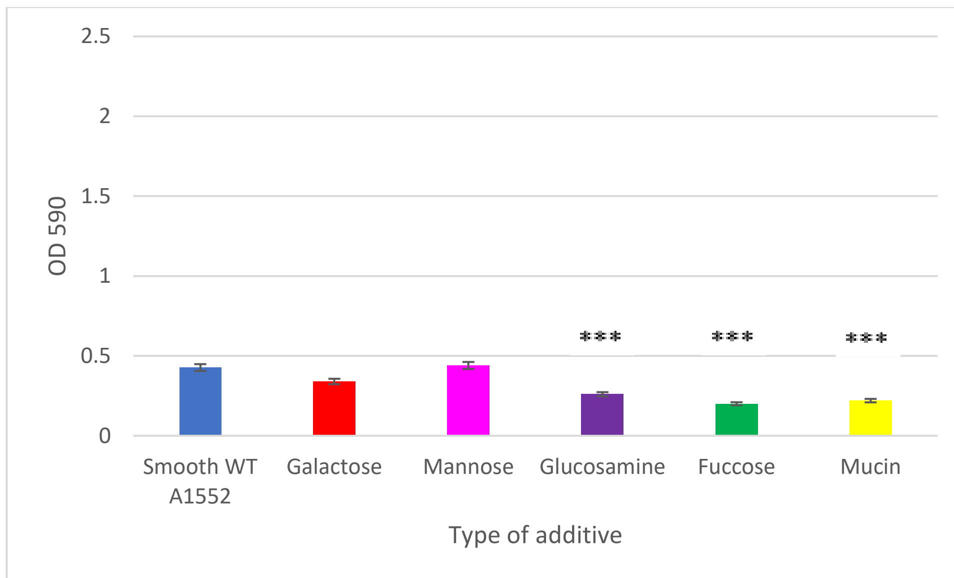

Figure 5.4.2. Biofilm production by *V. cholerae.* Amount of biofilm produced by strain A1552 smooth wild type in the presence of galactose, mannose, glucosamine, fucose and mucin. Each bar represents the mean of three replicates; standard error is indicated by error bars. *** equates to a p value of $p \leq 0.05$ when compared to the smooth and/or its rugose variant.

5.4.3 Effects of galactose, mannose, glucosamine, fucose and mucin on Vibrio cholerae strain A1552 rugose variant biofilm formation

Galactose, mannose, glucosamine, fucose and mucin (as control) were added to *V. cholerae* strain A1552 rugose variant to determine if these monosaccharides were able to inhibit biofilm formation.

Figure 5.4.3 show that there was no significant difference detected when mannose ($p \leq 0.2879$), and glucosamine ($p \leq 0.4020$) were added, as compared to un-supplemented cells. However, when galactose ($p \leq 0.0449$), fucose ($p \leq 0.0001$) and mucin ($p \leq 0.0001$) were added, there was a significant decrease in the amount of biofilm formed. This suggests that specific sugars, such as galactose, fucose and mucin may have an inhibitory effect on the *V. cholerae* strain A1552 rugose variant biofilm.

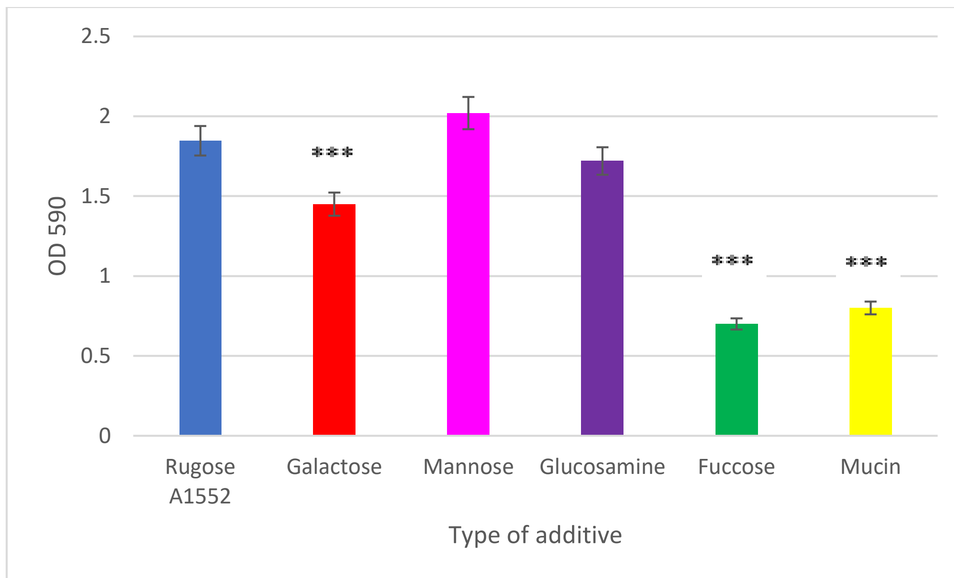

Figure 5.4.3. Biofilm production by *V. cholerae.* Amount of biofilm produced by strain A1552 rugose variant in the presence of galactose, mannose, glucosamine, fucose and mucin. Each bar represents the mean of three replicates; standard error is indicated by error bars. *** equates to a p value of $p \leq 0.05$ when compared to the smooth and/or its rugose variant.

5.4.4 Effects of galactose, mannose, glucosamine, fucose and mucin on Vibrio cholerae strain VPS mutant 4327 biofilm formation

Galactose, mannose, glucosamine, fucose and mucin (as control) were added to *V. cholerae* VPS mutant strain 4327 to determine if these monosaccharides were able to inhibit biofilm formation.

Figure 5.4.4 show that there was no significant difference detected when galactose ($p \leq 0.3375$) and glucosamine ($p \leq 0.0817$) were added, as compared to un-supplemented cells. However, when mannose ($p \leq 0.0272$), fucose ($p \leq 0.0061$) and mucin ($p \leq 0.0121$) were added, there was a significant decrease in the amount of biofilm formed. This suggests that specific sugars, such as mannose, fucose and mucin may have an inhibitory effect on the *V. cholerae* strain VPS mutant 4327 biofilm.

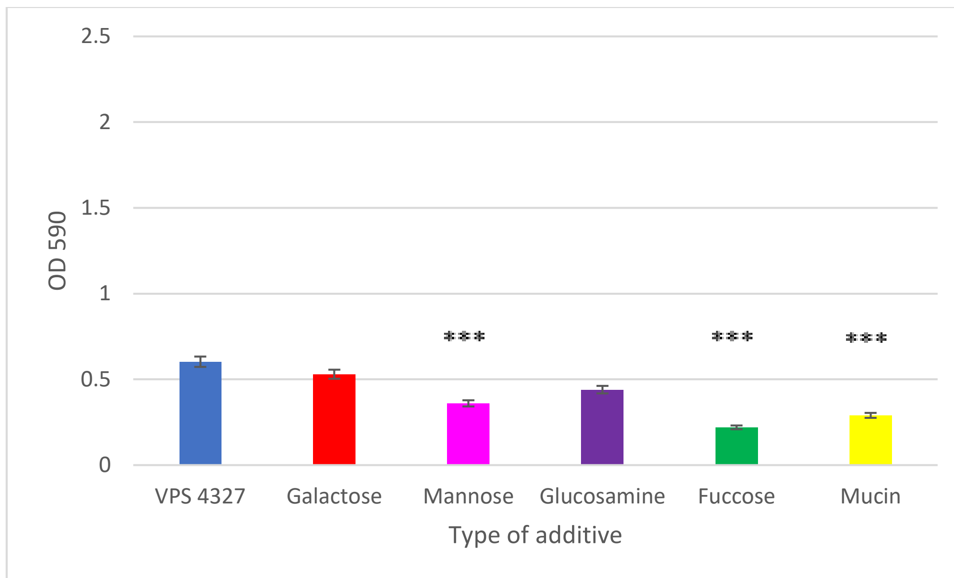

Figure 5.4.5. Biofilm production by *V. cholerae.* Amount of biofilm produced by strain 4327 VPS mutant in the presence of galactose, mannose, glucosamine, fucose and mucin. Each bar represents the mean of three replicates; standard error is indicated by error bars. *** equates to a p value of $p \leq 0.05$ when compared to the smooth and/or its rugose variant.

5.4.5 Effects of galactose, mannose, glucosamine, fucose and mucin on Vibrio cholerae strain LPS mutant 13565 biofilm formation

Galactose, mannose, glucosamine, fucose and mucin (as control) were added to *V. cholerae* strain 13565 LPS mutant to determine if these monosaccharides were able to inhibit biofilm formation.

Figure 5.4.5 show that when galactose ($p \leq 0.0001$), mannose ($p \leq 0.0385$), glucosamine ($p \leq 0.0002$), fucose ($p \leq 0.0001$) and mucin ($p \leq 0.0001$) were added, there was a significant decrease in the amount of biofilm formed. This suggests that specific sugars, such as galactose, mannose, glucosamine, fucose and mucin may all have an inhibitory effect on the *V. cholerae* strain LPS mutant 13565 biofilm.

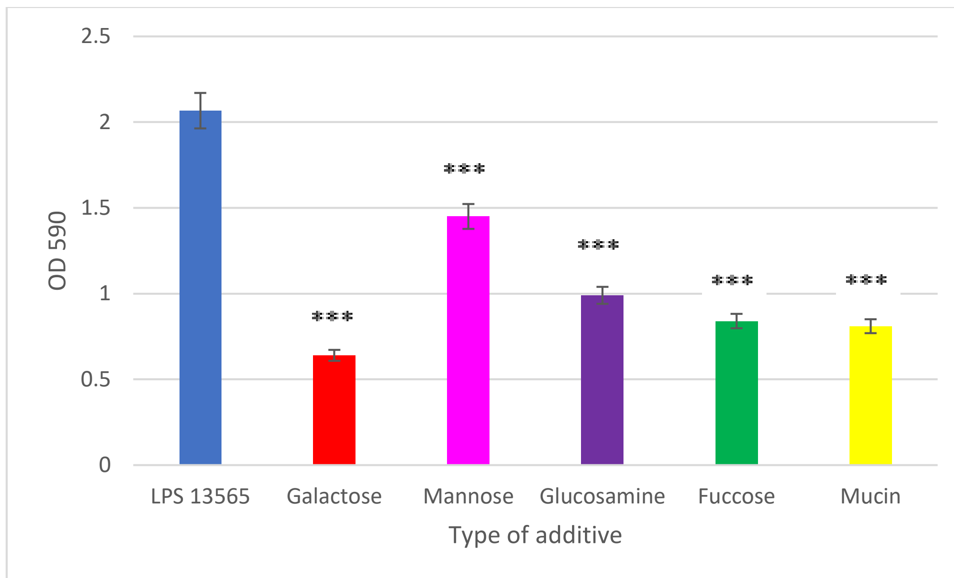

Figure 5.4.5. Biofilm production by *V. cholerae.* Amount of biofilm produced by strain 13565 LPS mutant in the presence of galactose, mannose, glucosamine, fucose and mucin. Each bar represents the mean of three replicates; standard error is indicated by error bars. *** equates to a p value of $p \leq 0.05$ when compared to the smooth and/or its rugose variant.

5.4.6 Effects of galactose, mannose, glucosamine, fucose and mucin on Vibrio cholerae strain biofilm matrix protein mutant 4329 biofilm formation

Galactose, mannose, glucosamine, fucose and mucin (as control) were added to the *V. cholerae* biofilm matrix protein mutant strain 4329 to determine if these monosaccharides were able to inhibit biofilm formation.

Figure 5.4.6 show that when galactose ($p \leq 0.0266$), mannose ($p \leq 0.0424$), glucosamine ($p \leq 0.0080$), fucose ($p \leq 0.0213$) and mucin ($p \leq 0.0141$) were added, there was a significant decrease in the amount of biofilm formed. This suggests that specific sugars, such as galactose, mannose, glucosamine, fucose, and in addition, mucin, may have an inhibitory effect on the biofilm of the mutant *V. cholerae* strain 4329.

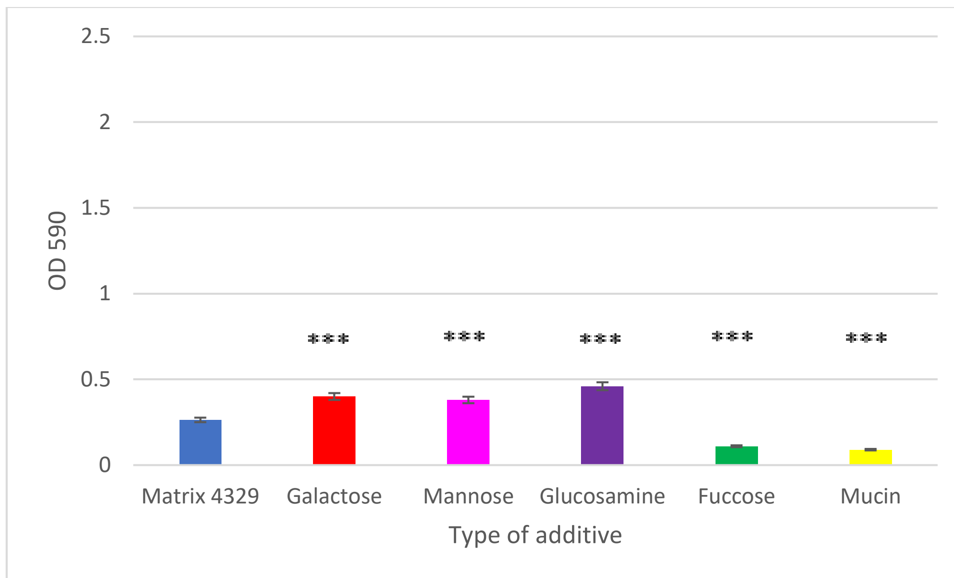

Figure 5.4.6. Biofilm production by *V. cholerae.* Amount of biofilm produced by strain 4329 in the presence of galactose, mannose, glucosamine, fucose and mucin. Each bar represents the mean of three replicates; standard error is indicated by error bars. *** equates to a p value of $p \leq 0.05$ when compared to the smooth and/or its rugose variant.

5.4.7 Effects of galactose and VPS on the biofilm of Vibrio cholerae mutants VPS 4327 and LPS 13565

Galactose and VPS were added to *V. cholerae* strains 4327 VPS, 13565 LPS and 4329 biofilm matrix protein mutants and biofilm formation was examined.

Figure 5.4.7 show that when VPS is added to the rugose variant in the presence of galactose, there is no significant increase ($p \geq 0.5$) in the biofilm formation. When VPS is added to the VPS 4327 mutant in the presence of galactose, there is no significant increase ($p \geq 0.1$) in the biofilm formation. When VPS is added to the LPS 13565 mutant in the presence of galactose, there is no significant increase ($p \geq 0.1$) in the biofilm formation. This suggests that the addition of VPS may not have a significant effect on the biofilm of the *V. cholerae* mutants 4327 and 13565.

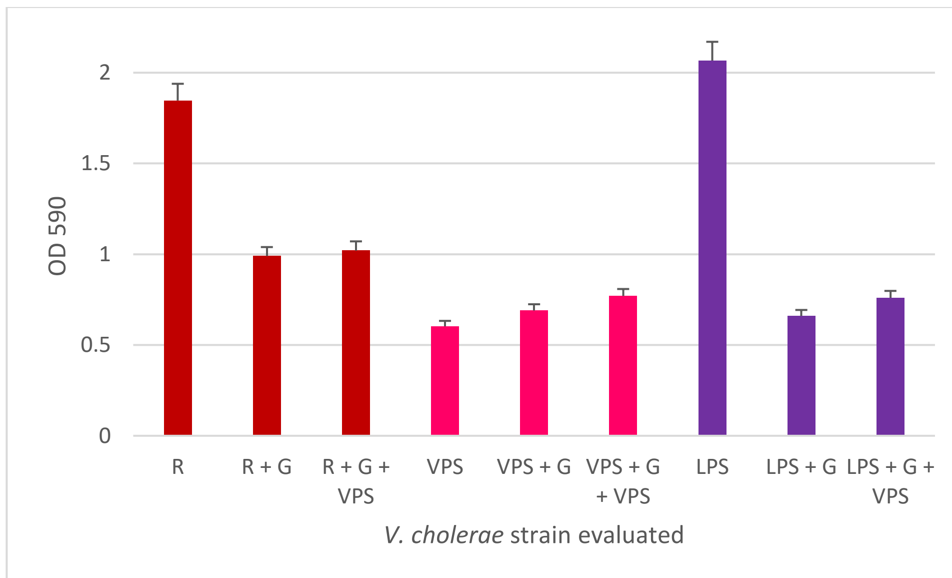

Figure 5.4.7. Biofilm production by *V. cholerae.* Amount of biofilm produced by strains in the presence of 50 mM of galactose and VPS (10 ug/mL). Each bar represents the mean of three replicates; standard error is indicated by error bars. *** equates to $p \leq 0.05$. R (rugose variant strain). R + G (rugose variant strain + 50 mM of galactose), R + G + VPS (rugose variant strain + 50 mM of galactose + 10 ug/mL of VPS), VPS (VPS mutant 4327 strain), VPS + G (VPS mutant 4327 strain + 50 mM of galactose), VPS + G + VPS (VPS mutant 4327 strain + 50 mM of galactose + 10 ug/mL of VPS), LPS (LPS mutant 13565 strain), LPS + G (LPS mutant 13565 strain + 50 mM of galactose), LPS + G + VPS (LPS mutant 13565 strain + 50 mM of galactose + 10 ug/mL of VPS).

5.4.8 Effects of galactose and LPS on the biofilm of Vibrio cholerae mutants VPS 4327 and LPS 13565

In this study, we investigated whether adding LPS could counter the effect of galactose on *V. cholerae* biofilm formation.

Galactose and LPS were added to *V. cholerae* strains 4327 VPS, 13565 LPS and 4329 biofilm matrix protein mutants and biofilm formation was examined.

Figure 5.4.8 show that when LPS is added to the rugose variant in the presence of galactose, there is no significant increase ($p \geq 0.5$) in the biofilm. When LPS is added to the VPS 4327 mutant in the presence of galactose, there is a significant increase ($p \leq 0.01$) in the biofilm. When LPS is added to the LPS 13565 mutant in the presence of galactose, there is no significant increase ($p \geq 0.5$). This suggests that the addition of LPS to the 4327 VPS mutant may have an effect on the biofilm.

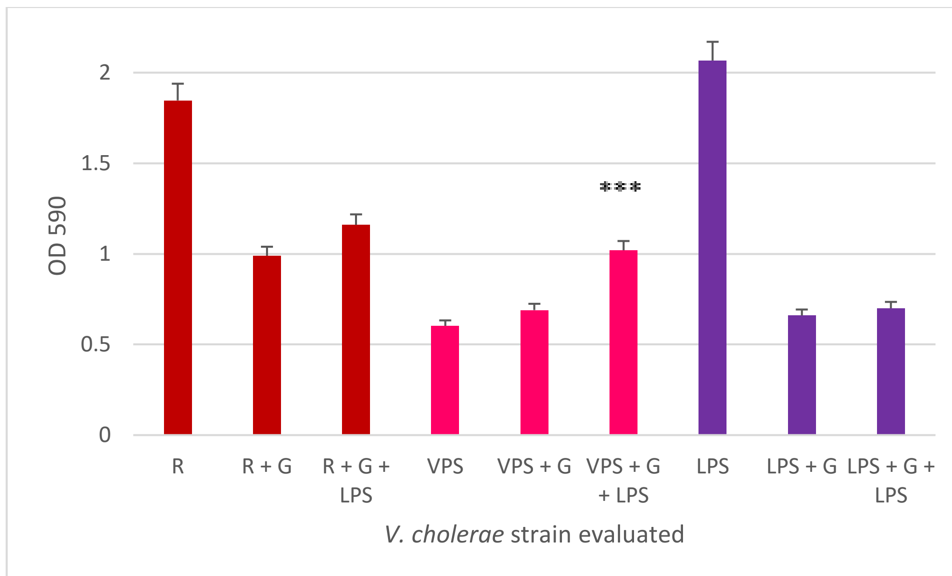

Figure 5.4.8. Biofilm production by *V. cholerae.* Amount of biofilm produced by strains in the presence of 50 mM of galactose and LPS (10 ug/mL). Each bar represents the mean of three replicates; standard error is indicated by error bars. *** equates to $p \leq 0.05$. R (rugose variant strain). R + G (rugose variant strain + 50 mM of galactose), R + G + LPS (rugose variant strain + 50 mM of galactose + 10 ug/mL of LPS), VPS (VPS mutant 4327 strain), VPS + G (VPS mutant 4327 strain + 50 mM of galactose), VPS + G + LPS (VPS mutant 4327 strain + 50 mM of galactose + 10 ug/mL of LPS), LPS (LPS mutant 13565 strain), LPS + G (LPS mutant 13565 strain + 50 mM of galactose), LPS + G + LPS (LPS mutant 13565 strain + 50 mM of galactose + 10 ug/mL of LPS).

5.4.9 Analysis and discussion

Glycan array analysis of the VPS mutant 4327, the LPS mutant 13565 and the biofilm matrix protein mutant 4329 showed that all 3 stains had severely reduced binding to most of the simple glycans, when compared to A1552 smooth wild type and its rugose variant. Thus, it was decided to test whether saccharides caused an increase or decrease in the biofilm forming capabilities of these strains.

Overall, fucose and mucin were the only two additives that consistently inhibited the biofilm formation of the A1552 wild type strain, its rugose variant and the VPS, LPS and biofilm matrix protein mutants. The importance of these results is that these 2 monosaccharides reduced the amount of biofilm formed for all tested strains. The biofilm is important for the infectivity, transmission, and environmental persistence of *V. cholerae* (Corfù et al., 1992., Silva & Benitez, 2016). The biofilm allows for the bacterium to attach to host cells and reproduce in a relatively safe environment. This then leads to the secretion of virulence factors and eventual disease progression (Garcia-Peñarrubia et al., 2002; Ramamurthy et al., 2020). Thus, a reduction in the biofilm formation capabilities caused by these monosaccharides may cause *V. cholerae* cells to become more susceptible to environmental pressures and host immune defences, since they have reduced protection from the biofilm.

Glucosamine, along with fucose and mucin, was able to reduce the amount of biofilm formed by the A1552 wild type strain. This is important because these monosaccharides are able to inhibit a *V. cholerae* strain without any mutations, thus this might occur naturally in the environment. This is significant as these monosaccharides might be used as inhibitors of *V. cholera* wild type strains. A possible mechanism by which these monosaccharides exert their effect on the biofilm formation may involve competitive exclusion or competitive inhibition, but further research is needed before any conclusions can be made.

The rugose variant is a VPS-over-expressing variant of the A1552 wild type strain. Thus, the rugose variant overproduces biofilm. VPS is always produced, and its role is to aid in the formation of the three-dimensional structure of the biofilm matrix (Teschler, 2015). Galactose, along with fucose and mucin, was able to inhibit the biofilm formation of the rugose variant, while glucosamine was not able to inhibit the biofilm formation of the rugose variant, when compared to the wild type. This is important because these 3 monosaccharides are able to inhibit a *V. cholerae* strain with a much larger and thicker biofilm compared to the wild type. This is significant as these monosaccharides may have additional inhibitory effects on the biofilm formation of *V. cholerae* strains.

Mannose, along with fucose and mucin, were able to reduce the amount of biofilm formed for the 4327 VPS mutant, while the 13565 LPS mutant's biofilm formation was inhibited by galactose, mannose, glucosamine, fucose and mucin. This is important because these monosaccharides are able to inhibit a *V. cholerae* strain's amount of biofilm formed with a mutation to either the VPS or LPS of *V. cholerae*. Further study into the effects of these monosaccharides might provide insight into the protective involvement that VPS might have against monosaccharide inhibition of the biofilm formation. Mannose, which did not affect the biofilm formation of the wild type, did reduce the amount of biofilm formed in the VPS mutant, while galactose and mannose did reduce the amount of biofilm formed in the LPS mutant.

5.5 Analysis of adhesion and invasion by *Vibrio cholerae* strains and their mutants

The human epithelial cell line Caco-2 (Human colon carcinoma-derived) (American Type Culture Collection, HTB 37) was originally derived from a human colon adenocarcinoma and is widely employed in studies of pathogen-host cell interactions because of its ability to form well-differentiated cell monolayers. It has long been widely used as an in vitro intestinal model (Negoro, 2021). The cell line is very similar to small intestinal enterocytes with respect to their structure, brush border enzymes, and time courses of differentiation. Due to this fact, they are excellent in testing the adhesion and invasion capabilities of intestinal pathogens.

In this study, we compared the adhesion of *V. cholerae* A1552 smooth wild type strain, its rugose variant and the VPS 4327, LPS 13565 and biofilm matrix protein 4329 mutants to Caco-2 cells.

5.5.1 *The involvement of VPS, LPS and biofilm matrix proteins of Vibrio cholerae in adhesion to caco-2 cells*

To understand the involvement of VPS, LPS and the biofilm matrix proteins in the adhesion of *V. cholerae*, an adherence assay using Caco-2 cells was performed by leaving *V. cholerae* cells in the presence a Caco-2 monolayer over a period of 4 hours. The percentage of adherence was then calculated for both the wild type and mutant strains.

A significant increase was detected in the adherence of the rugose variant strain when compared to the A1552 smooth wild type ($p \leq 0.0001$) (Figure 5.5.1). A significant decrease in the adherence was detected in all 3 mutant strains (VPS mutant 4327, LPS mutant 13565, biofilm matrix protein mutant 4329), when compared to the rugose variant, their progenitor ($p \leq 0.0001$). All 3 strain showed similar levels of adherence comparable with the A1552 smooth wild type.

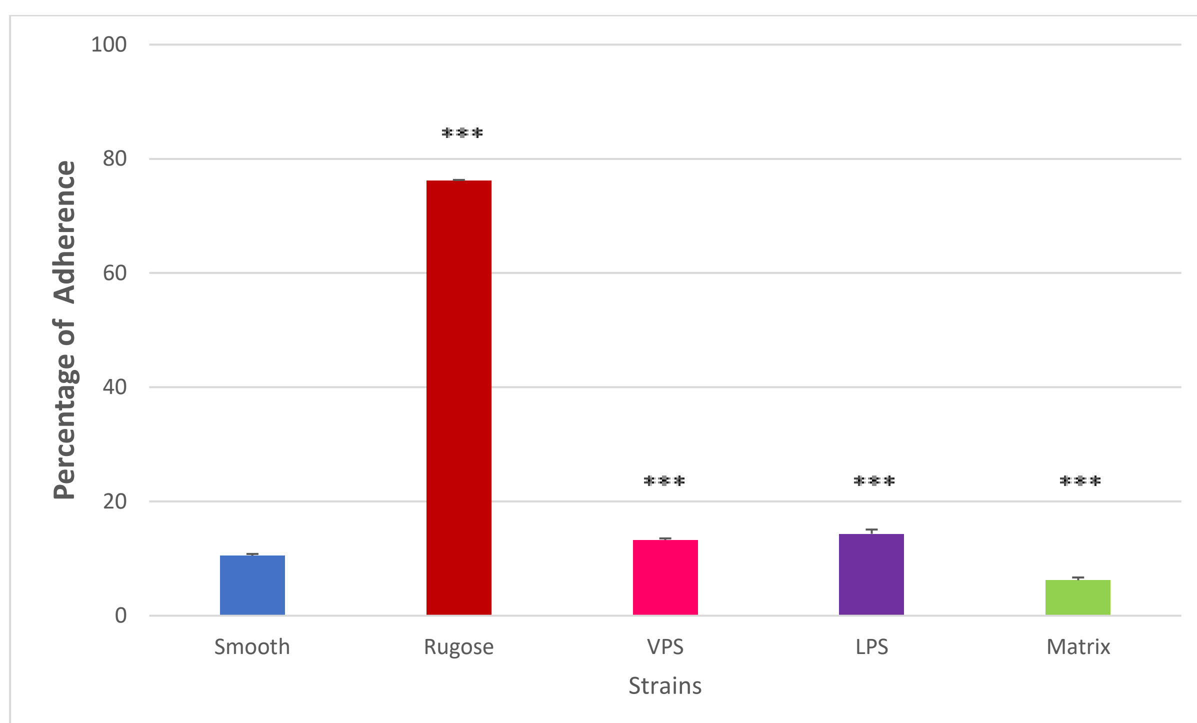

Figure 5.5.1: *V. cholerae* adherence to Caco-2 cells. Each value indicates the mean ± SEM from two independent experiments and three replicates (n = 3). * Indicates $p \leq 0.05$ as a significant difference.

5.5.2 *Analysis and discussion*

Overall, the rugose variant was found to have significantly higher adhesion to Caco-2 cells when compared to A1552 smooth wild type. This may be due to the overproduction of VPS, which is involved with biofilm formation and adhesion as supported by (Chowdhury, 2016).

We report that all 3 mutants (VPS 4327, LPS 13565 and biofilm matrix protein 4329) were found to have a significant reduction in adhesion to Caco-2 cells when compared to the rugose variant.

Chapter 6

General discussion

This study reports that the 2 environmental strains (M552 (O103) and M1566 (O114)) and the 2 clinical strains (M1575 (O1 El-Tor) and M2559 (O2)), all naturally produced more biofilm when compared to the amount of biofilm formed by the A1552 wild type, which is itself a known pathogenic strain. All 4 strains showed very similar levels of biofilm formation. Both environmental and clinical strains also had different adherence levels to Caco-2 cells. As such, no association for biofilm formation and Caco-2 cell adhesion between clinical and environmental strains could be drawn. However, a greater amount of biofilm is associated with greater protection from both environmental pressures and host defences (Lizárraga-Partida, 2009). Thus, it is not unexpected that we see more natural biofilm produced in strains that are subject to greater external pressures and harsher conditions. It is possible that screening of a large number of strains would have identified an association (or lack thereof) between biofilm formation, adherence and disease or survival in aquatic environments.

If we did find such an association, it could mean that strains from both clinical and environmental settings are capable of increased biofilm formation and increased adherence when compared to the A1552 wild type pathogenic strain, which has been linked with increased survivability in both the environment and host settings (Lizárraga-Partida, 2009). This may lead to a greater number of surviving pathogenic cells and a greater binding of *V. cholerae* cells to host cells (Wolska, 2016), which are capable of more easily causing disease (Chac et al., 2021). An increase in biofilm formation may also provide greater protection against host defences, while a greater number of successfully binding pathogenic cells are capable of more easily forming biofilms, and thus have a greater chance of survival and disease progression (Silva & Benitez, 2016). This, in turn, may have detrimental effects on the host.

If no such association exists, then the increased biofilm formation of clinical and environmental strains may not be involved in the increased survivability in both environmental and clinical settings and that the increased adherence of clinical and environmental strains is not involved in the increased survivability in both environmental and host settings.

Such an association would be used to further investigate how increased biofilm formation enhances environmental and host survival and how increased adherence could enhances bacterial pathogenicity.

Further study may include testing more environmental and clinical strains to try and discern a pattern in the biofilm formation capabilities and adherence to Caco-2 cells between environmental and clinical strains. Another avenue for research might involve NMR spectroscopy to determine the biofilm structures and protein ligand-interactions found in different environmental and clinical strains, as shown by (Monaco, et al., 2020).

In this study, we found a decrease in the extent of binding of both simple and complex glycans for the 4327 VPS, 13565 LPS and 4329 biofilm matrix protein mutant strains when compared

to the A1552 wild type pathogenic strain. Thus, there could be an association between VPS, LPS, and biofilm matrix proteins and their ability to bind to glycans.

This means that the mutant strains have lost the ability to interact with most glycans. Since glycan binding is important in the adhesion of *V. cholerae* cells to host cells (Silva & Benitez, 2016, De et al., 2018), a reduction in the binding could result in an overall reduction in biofilm formation. This will directly impact the ability of *V. cholerae* to cause disease. This suggests that VPS, LPS and the biofilm matrix proteins are extremely important in the binding of *V. cholerae* cells to these simple and complex glycans, as without them, the binding is either significantly reduced or entirely absent.

Future study may be directed towards using NMR spectroscopy to identify the structural components of VPS, LPS and biofilm matrix proteins. Further NMR spectroscopy may also reveal the structure of specific host glycans and how they interact with VPS, LPS and the biofilm matrix proteins (Monaco, et al., 2020). Previous NMR spectroscopy has already shown that delipidated fragments of the LPS of *V. cholerae O139* (OSPc) consists of the hex saccharide O-antigen being attached to the core oligosaccharide. However, the finer structural details, such as the location of the O-acetyl group, the position where the OSP is connected to the core oligosaccharide and the ring size in the anhydro Kdo group has not been agreed upon in the literature until recently (Xu et al. 2019). These gaps have since been filled based on extensive NMR study (Perepelov et al. 2019; Xu et al. 2019).

According to Xu (2019), the OSPc conjugate was present in patients recovering from cholera caused by the O139 serotype but not from patients recovering from the O1 serotype. Thus, NMR may be used in the future to identify the binding sites between LPS and relevant host glycans, and how this might play a role in the pathogenicity of *V. cholerae*.

It was reported here that there was a reduction in the biofilm auto-aggregation of the 13565 LPS mutant strain when in the presence of galactose. Thus, there may be an association between auto-aggregation and the presence of monosaccharides. This could mean that galactose has the ability to reduce the auto-aggregation the 13565 LPS mutant strain. Since LPS is involved in the adherence of *V. cholerae* cells to host cells (Teschler, 2015), a reduction in auto-aggregation could result in less adherence to the host cells, which may impact the number of successful pathogenic cells capable of causing disease.

Further study is needed to determine the involvement that galactose has in auto-aggregation. A possible mechanism for galactose may involve testing by competitive exclusion or adherence inhibition (Hu et al., 2010).

In this study, we have also found a difference in the biofilm formation between the A1552 wild type pathogenic strain, its rugose variant and the 4327 VPS, 13565 LPS and 4329 biofilm matrix protein mutant strains when certain monosaccharides were added. Thus, there could be an association between biofilm formation and the presence of monosaccharides. This means that the presence of monosaccharides has the ability to either reduce or increase the biofilm formation of *V. cholerae* strains. The biofilm is important for the infectivity,

transmission, and environmental persistence of *V. cholerae* (Corfù et al., 1992., Silva & Benitez, 2016). The biofilm allows for the bacterium to reproduce in a relatively safe environment. This then leads to the secretion of virulence factors and eventual disease progression (Garcia-Peñarrubia et al., 2002; Ramamurthy et al., 2020). Thus, a reduction in the biofilm formation capabilities caused by these monosaccharides may cause *V. cholerae* cells to become more susceptible to environmental pressures and host immune defences, since they have reduced protection from the biofilm. Thus, VPS may have a protective involvement against mannose's inhibitory effects on the biofilm formation, while LPS may have a protective involvement against galactose and mannose's inhibitory effects on the biofilm formation.

The 4329 biofilm matrix protein mutant's biofilm formation was stimulated by galactose, mannose and glucosamine, while fucose and mucin inhibited the biofilm formation of the strain. This is important because galactose, mannose and glucosamine were able to increase a *V. cholerae* strain's amount of biofilm formed with a mutation to the biofilm matrix proteins of *V. cholerae*. The amount of biofilm formed was increased in the presence of these monosaccharides, which was not seen in any other strain. In the A1552 wild type strain, glucosamine had the opposite effect and decreased the amount of biofilm formed. On the other hand, fucose and mucin inhibited the biofilm formation when the biofilm matrix proteins were mutated. Further study into these monosaccharides might provide insight into the protective involvement that the biofilm matrix proteins might have against monosaccharide inhibition of the biofilm formation. Thus, the biofilm matrix proteins might modulate biofilm production.

There is a lack of research around the involvement that monosaccharides have in biofilm formation. Another avenue for research might involve NMR spectroscopy to determine the biofilm structures and protein ligand-interactions found in the VPS, LPS and biofilm matrix protein mutants, as shown by (Monaco, et al., 2020).

It was reported here that there was a difference in the adherence to Caco-2 cells between the A1552 wild type pathogenic strain and the 4327 VPS, 13565 LPS and 4329 biofilm matrix protein mutant strains. Thus, there could be an association between the presence of VPS, LPS and the biofilm matrix proteins in *V. cholerae* and their adherence to Caco-2 cells. Therefore, VPS, LPS and the biofilm matrix proteins may be involved in the adherence process of *V. cholerae* cells to host cells. This confirms the Glycan Array results, which showed that the mutant strains have significantly reduced binding and would be expected to have reduced adhesion to cell surfaces. All 3 mutant strains appeared to show similar results to the A1552 smooth wild type strain phenotype in terms of binding to Caco-2 cells.

Since *V. cholerae* enters the human body via oral administration (Ali, 2018), it must interact with the intestine and its intestinal epithelial cells in order to cause disease. Thus, the Caco-2 monolayer gives a more realistic approach to a human intestinal setting. And since they are derived from human epithelial cells, they express the same glycans as normal epithelial cells (De et al. 2018). Thus, they are the right model to use for *V. cholerae* cells.

V. cholerae cells must bind to the surface of human epithelial cells before they can downregulate genes required for motility and upregulate genes required for biofilm production (Silva & Benitez, 2016). Quorum sensing then leads to exopolysaccharide secretion and biofilm formation (Yildiz & Visick, 2009). When the bacterium becomes established, the binding becomes irreversible (Wolska, 2016). Thus, if adhesion was to be reduced, less *V. cholerae* cells would successfully colonise the host gut mucosa. This may lead to a better prognosis for the host, especially as future investigations may use NRM spectroscopy to look into the structures of VPS, LPS and biofilm matrix proteins and their interactions with host glycans (Monaco, et al., 2020).

Overall, the findings of this thesis highlight the importance of VPS, LPS and the matrix proteins RmbA, RmbC and Bap1, and the role they may play in *V. cholerae* biofilm production. They also reveal how different monosaccharides, especially galactose, interact with these mutant strains and how the biofilm might be increased or reduced.

This thesis provides novel data to the literature and bridges the gap between VPS, LPS, the matrix proteins and how they bind to different glycans through glycan arrays and in-vivo on Caco-2 cells.

With new advances in NMR spectroscopy and Caco-2 cell technology, the possibility is open for developing and evaluating conjugate vaccines targeting *V. cholerae* and its biofilm production.

Chapter 7

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Chapter 8

Supplementary data

Table S1. Glycans bound by *V. cholerae* strains (*V. cholerae* A1552 smooth wildtype, Rugose variant, and Matrix mutant (RΔrbmAΔrbmCΔbap1)) in glycan array analysis. The table shows result of glycan-binding by whole-cell *V. cholerae* strains. Glycans are clustered into classes based on their respective terminal sugars.

Smooth WT	**Rugose**	**Matrix mutant**
MONOSACCHARIDES & DISACCHARIDES		
• Fucα-sp3 • Glcα-sp3 • GlcNAcβ-sp3 • Rhaα-sp3 • Manα-sp3 • 6-H2PO3Manα-sp3 • 3-O-Su-Galβ-sp3 • 3-O-Su-GlcNAcβ-sp3 • 3-O-Su-GalNAcα-sp3 • Neu5Gcα-sp3 • GlcNAcβ-sp4 • 6-H2PO3Glcβ-sp4 • Neu5Acα-sp9 • GlcN(Gc)β-sp4 • 9-NAc-Neu5Acα-sp3 • Galβ1-3GlcNaAcβ-sp3 (Le^{c}) • Galβ1-3GalNAcβ-sp3 • Galα1-3GlcNAcβ-sp3 • Galβ1-3Galβ-sp3 • GalNAcβ1-3Galβ-sp3	• Rhaα-sp3 • Galα-sp3 • Fucα-sp3 • Glcα-sp3 • Manα-sp3 • 9-NAc-Neu5Acα-sp3 • 3-O-Su-GalNAcα-sp3 • 6-H2PO3Manα-sp3 • 3-O-Su-Galβ-sp3 • GlcN(Gc)β-sp4 • GlcNAcβ-sp4 • Neu5Gcα-sp3 • Neu5Acα-sp9 • Galβ1-3GlcNaAcβ-sp3 (Le^{c}) • Neu5Gcα2-6GalNAcα-sp3 • GlcNAcβ1-4GlcNAcβ-Asn (chitobiose-Asn) • Galα1-6Glcβ-sp4 • Galα1-4Galβ1-4GlcNAc-sp2 • Galα1-3GalNAcα-sp3	• Galβ1-3Galβ-sp3

• GalNAcα1-3Galβ-sp3 • GlcNAcβ1-4GlcNAcβ-Asn (chitobiose-Asn) • Galβ1-3GalNAcα-sp3 • Galα1-3GalNAcα-sp3 • Galα1-3Galβ-sp3 • Galβ1-2Galβ-sp3 • Galβ1-4Galβ-sp4 • Fucα1-4GlcNAcβ-sp3 (Le^c) • Galβ1-4(6-O-Su)GlcNAcβ-sp3 • GalNAcβ1-4(6-O-Su)GlcNAcβ-sp3 • 6-O-Su-GalNAcβ1-4GlcNAcβ-sp3 • GlcAβ1-3Galβ-sp33-O-Su-GalNAcβ1-4(3-O-Su)-GlcNAcβ-sp3 • Neu5Acα2-8Neu5Acα2-sp3 • 6-O-Su-Galβ1-3(6-O-Su)GlcNAcβ-sp2 • 4-O-Su-Galβ1-4GlcNAcβ-sp3 • 3,4-O-Su_2-Galβ1-4GlcNAcβ-sp3 • 6-O-Su-Galβ1-3GalNAcα-sp3 • 4,6-O-Su_2-Galβ1-4GlcNAcβ-sp2 • 6-O-Su-Galβ1-4Glcβ-sp2 • 3-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp2 • Neu5Gcα2-6GalNAcα-sp3 • Neu5Acα2-3GalNAcα-sp3 • 6-O-Su-GalNAcβ1-4-(3-O-Su)GlcNAcβ-sp3	• 3-O-Su-GalNAcβ1-4(3-O-Su)-GlcNAcβ-sp3 • Galβ1-4GlcNAcβ-sp3 • Galβ1-3GalNAcα-sp3 • Fucα1-3GlcNAcβ-sp3 • Galβ1-3Galβ-sp3 • GalNAcβ1-3Galβ-sp3 • GalNAcα1-3Galβ-sp3 • Neu5Acα2-8Neu5Acα2-sp3 • 6-O-Su-Galβ1-3(6-O-Su)GlcNAcβ-sp2 • 4-O-Su-Galβ1-4GlcNAcβ-sp3 • 3,4-O-Su_2-Galβ1-4GlcNAcβ-sp3 • 6-O-Su-Galβ1-3GalNAcα-sp3 • 4,6-O-Su_2-Galβ1-4GlcNAcβ-sp2 • 6-O-Su-Galβ1-4Glcβ-sp2 • 3-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp2 • Neu5Gcα2-6GalNAcα-sp3 • Neu5Acα2-3GalNAcα-sp3 • 6-O-Su-GalNAcβ1-4-(3-O-Su)GlcNAcβ-sp3 • GlcNAcβ1-4(6-O-Su)GlcNAcβ-sp2 (6-O-Su-chitobiose) • 6-O-Su-GalNAcβ1-4-(3-O-Su)GlcNAcβ-sp3	• Neu5Acα2-3GalNAcα-sp3

<table>
<tr>
<td>• GlcNAcβ1-4(6-O-Su)GlcNAcβ-sp2 (6-O-Su-chitobiose)
• Galβ1-4(6-O-Su)Glcβ-sp2
• GlcAβ1-3GlcNAcβ-sp3
• 6-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp2
• 4,6-O-Su_2-GalNAcβ1-4GlcNAcβ-sp3
• 3-O-Su-Galβ1-4(6-O-Su)Glcβ-sp2</td>
<td>• Galβ1-4(6-O-Su)Glcβ-sp2
• GlcAβ1-3GlcNAcβ-sp3
• 6-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp2
• 4,6-O-Su_2-GalNAcβ1-4GlcNAcβ-sp3</td>
<td></td>
</tr>
<tr>
<td colspan="3">TRISACCHARIDES & TETRASACCHARIDES</td>
</tr>
<tr>
<td>• GlcNAcβ1-4Galβ1-4GlcNAcβ-sp2
• Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ-sp3
• Neu5Acα2-3Galβ1-3GalNAcα-sp3
• Neu5Acα2-3Galβ1-4GlcNAcβ-sp3
• Galα1-3(Neu5Acα2-6)GalNAcα-sp3
• Galβ1-3Galβ1-4GlcNAcβ-sp4
• Neu5Acα2-6Galβ1-3GlcNAc-sp3
• GlcNAcβ1-3(GlcNAcβ1-6)GalNAcα-sp3
• Neu5Gcα2-3Galβ1-3GlcNAcβ-sp3
• 3-O-Su-Galβ1-3(Fucα1-4)GlcNAcβ-sp3 (Su-Le^a)
• Fucα1-2(Galα1-3)Galβ-sp3
• Neu5Acα2-3-(6-O-Su)Galβ1-4GlcNAcβ-sp3</td>
<td>• GlcNAcβ1-4Galβ1-4GlcNAcβ-sp2
• Fucα1-2(GalNAcα1-3)Galβ-sp3
• GlcNAcβ1-3Galβ1-4Glcβ-sp2
• Galβ1-2Galα1-4GlcNAcβ-sp4
• Galβ1-3(Fucα1-4)GlcNAcβ-sp3 (Le^a)
• Neu5Acα2-6Galβ1-3(6-O-Su)GlcNAc-sp3
• GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3
• Neu5Acα2-3Galβ1-4Glcβ-sp3
• Neu5Acα2-3Galβ1-4GlcNAcβ-sp3
• GalNAcβ1-4Galβ1-4Glcβ-sp3
• Galα1-3(Neu5Acα2-6)GalNAcα-sp3</td>
<td>• Galβ1-2Galα1-4GlcNAcβ-sp4</td>
</tr>
</table>

• GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2 • Neu5Gcα2-3Galβ1-4GlcNAcβ-sp3 • GlcNAcβ1-2Galβ1-3GalNAcα-sp3 • Neu5Acα2-6Galβ1-4Glcβ-sp2 • Neu5Acα2-3Galβ1-3-(6-O-Su)GalNAcβ-sp3 • GlcNAcβ1-3Galβ1-3GalNAcα-sp3 • Neu5Acα2-3Galβ1-4Glcβ-sp4 • 9-NAc-Neu5Acα2-6Galβ1-4GlcNAcβ-sp3 • GlcNAcβ1-3Galβ1-4Glcβ-sp2 • Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ-sp3 • Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAcα-sp3 • 3-O-SuGalβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 • Neu5Acα2-3(GalNAcβ1-4)Galβ1-4Glcβ-sp2 (GM2) • Fucα1-2(GalNAcα1-3)Galβ1-3GalNAcα-sp3 (Blood Group A (type 3) • Fucα1-2(Galα1-3)Galβ1-4GlcNAcβ-sp3 Blood Group B (type 2) • Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-sp3 Asialo-GM1 • Fucα1-3(Neu5Acα2-3Galβ1-4)6-O-Su-GlcNAcβ-sp3	• GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 • Galβ1-3Galβ1-4GlcNAcβ-sp4 • Neu5Acα2-3Galβ1-4-(6-O-Su)GlcNAcβ-sp3 • Neu5Acα2-6Galβ1-3GlcNAc-sp3 • GlcNAcβ1-3(GlcNAcβ1-6)GalNAcα-sp3 • Galβ1-4GlcNAcβ1-6GalNAcα-sp3 • Neu5Gcα2-6Galβ1-4GlcNAcβ-sp3 • Neu5Gcα2-3Galβ1-3GlcNAcβ-sp3 • Fucα1-3(Galβ1-4)GlcNAcβ-sp3 (Le^x) • Galβ1-3GalNAcβ1-3Gal-sp4 • Galβ1-4GlcNAcβ1-3GalNAcα-sp3 • Neu5Acα2-6Galβ1-3(6-O-Su)GlcNAc-sp3 • Neu5Acα2-3Galβ1-3-(6-O-Su)GalNAcβ-sp3 • Neu5Gcα2-3Galβ1-4GlcNAcβ-sp3 • Neu5Acα2-6Galβ1-4-(6-O-Su)GlcNAcβ-sp3 • GlcNAcβ1-2Galβ1-3GalNAcα-sp3 • Galβ1-4GlcNAcβ1-6GalNAcα-sp3	

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<td>• Galβ1-3GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2
• Galβ1-3GlcNAcα1-3Galβ1-4GlcNAcβ-sp3
• Fucα1-2(GalNAcα1-3)Galβ1-4GlcNAcβ-sp3 Blood Group A (type 2)
Fucα1-3(Fucα1-2Galβ1-4)GlcNAcβ-sp3 (Le^{y})</td>
<td>• 9-NAc-Neu5Acα2-6Galβ1-4GlcNAcβ-sp3
• GlcNAcβ1-3Galβ1-4Glcβ-sp2
• Neu5Acα2-6Galβ1-4Glcβ-sp2
• Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ-sp3
• Galβ1-3GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2
• 4-O-SuGalβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3
• Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAcα-sp3
• Galα1-3Galβ1-4GlcNAcβ1-3Galβ-sp3
• Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3
• Galβ1-3GlcNAcα1-3Galβ1-4GlcNAcβ-sp3
• Fucα1-2(Galα1-3)Galβ1-3GalNAcβ-sp3 Blood Group B (type 4)
• Fucα1-2(Galα1-3)Galβ1-3GlcNAcβ-sp3 Blood Group B (type 1)
• Galβ1-3GlcNAcβ1-3Galβ1-3GlcNAcβ-sp3 (Le^{c})
• Neu5Acα2-3(GalNAcβ1-4)Galβ1-4Glcβ-sp2(GM2)
• Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-sp3 Asialo-GM1</td>
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	• Fucα1-2(GalNAcα1-3)Galβ1-3GalNAcα-sp3 Blood Group A (type 3) • Galβ1-3GlcNAcα1-6Galβ1-4GlcNAcβ-sp2 • Fucα1-3(Fucα1-2Galβ1-4)GlcNAcβ-sp3(Le^y) • Fucα1-3(Neu5Acα2-3(6-O-Su)Galβ1-4)GlcNAcβ-sp3 • Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAcα-sp3 • (Glcα1-4)$_4$β-sp4 • Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2 • GalNAcβ1-3Galα1-4Galβ1-4Glcβ-sp3	
PENTA-NONA SACCHARIDES		
• Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ-sp4 • Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc-sp2 • Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc-sp2 • GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glc-sp2 GD2 • (Galβ1-4GlcNAcβ1-3)3-sp3 • Lex1-6'(6'SLN1-3')Lac-sp4 • GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAcβ-sp2 • (A-GN-M)2-3,6-M-GN-GNβ-sp4 • Lex1-6'(Leb1-3')Lac-sp4 TFLNH	• Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp4 • Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-sp4 • Fucα1-3(Neu5Acα2-3 Galβ1-4)GlcNAcβ1-3Galβ-sp3 • GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glc-sp2 GD2 • (Glcα1-6)6β-sp4 • Lex1-6'(Leb1-3')Lac-sp4 TFLNH • (A-GN-M)2-3,6-M-GN-GNβ-sp4	

• (Glcα1-6)$_6$β-sp4 • Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ-sp4 • (GN-M)2-3,6-M-GN-GNβ-sp4 •	• Fucα1-3(Fucα1-2Galβ1-4)GlcNAcβ1-3Galβ1-4Glcβ-sp4 (Le^y-Lac) • (Neu5Acα2-8)2Neu5Acα2-3(GalNAcβ1-4)Galβ1-4Glc-sp2 GT2 • Fucα1-2Galβ1-3GlcNAc Blood Group H Type II Trisaccharide • Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp4 • Blood Group H type1Lac • Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp2 Blood Group H (type1) penta • (Galβ1-4GlcNAcβ1-3)3-sp3 • Lex1-6'(6'SLN1-3')Lac-sp4 • Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 • Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc-sp2 • Lex1-6'(Led1-3')Lac-sp4 • (Glcα1-6)5β-sp4 • Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ-sp4	
Terminal Galactose		
• Galβ1-4Gal • Galβ1-3GlcNAc • Galα1-4Galβ1-4Glc • Galβ1-3(Fucα1-2)Gal Blood Group B Trisaccharide	• Galβ1-4Gal • Galβ1-3GlcNAc • Galα1-4Galβ1-4Glc • Galβ1-3GlcNAcβ1-3Galβ1-4Glc	• Galβ1-4Gal • Galα1-4Galβ1-4Glc

• Galα1-3Galβ1-4Galα1-3Gal • Galβ1-3GalNAcβ1-4Galβ1-4Glc asialo GM1 • Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	• Galβ1-3GalNAcβ1-4Galβ1-4Glc asialo GM1 • Galα1-4Galβ1-4GlcNAc P1 antigen • Galα1-3(Fucα1-2)Galβ1-4(Fucα1-3)Glc • Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc • Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	
Fucosylated glycans		
• Fucα1-2Galβ1-4(Fucα1-3)GlcNAc LewisY (Le^{Y}) • Fucα1-2Galβ1-4Glc • Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc • Galβ1-4(Fucα1-3)GlcNAc • Galβ1-3(Fucα1-4)GlcNAc Lewis A (Le^{a}) • Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc • Galβ1-4(Fucα1-3)Glc Galβ1-4(Fucα1-3)GlcNAcβ1-3Gal (Le^{x}) • GalNAcα1-3(Fucα1-2)Gal Blood Group A trisaccharide • Galβ1-4(Fucα1-3)GlcNAcβ1-3Gal • Galα1-3(Fucα1-2)Galβ1-4(Fucα1-3)Glc Blood Group B pentasaccharide • Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc	• Fucα1-2Galβ1-4(Fucα1-3)GlcNAc LewisY (Le^{Y}) • Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc • Galβ1-4(Fucα1-3)Glc • Fucα1-2Galβ1-4Glc • Galβ1-4(Fucα1-3)GlcNAcβ1-3Gal (Le^{x}) • GalNAcα1-3(Fucα1-2)Gal Blood Group A trisaccharide • Galβ1-3(Fucα1-4)GlcNAc Lewis A (Le^{a}) • Fucα1-2Galβ1-3GalNAcβ1-3Gal Blood group H antigen tetraose type 4 • Galβ1-4(Fucα1-3)GlcNAc • Fucα1-2Galβ1-3(Fucα1-4)GlcNAc Lewis B (Le^{b}) tetrasaccharide • GalNAcα1-3(Fucα1-2)Galβ1-4(Fucα1-3)Glc	• Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc

	Blood group A pentasaccharide • Galα1-3(Fucα1-2)Galβ1-4(Fucα1-3)Glc Blood Group B pentasaccharide	
Mannosyl containing glycans		
• Manα1-2Man	• Manα1-3(Manα1-6)Manβ-sp4 • Manα1-2Man • Manα1-6Man	• Manα1-3(Manα1-6)Manβ-sp4 • Manα1-6Man
Glycosaminoglycans - high and low molecular weight		
• ΔUA-GlcNAc (Delta Di-HA) • HA-16 4.9mM • ((±2S)GlcA/IdoAα/b1-3(±4S)GalNAcβ1-4)n (n<250) • HA - 4 10mM • HA 190000 da 2.5 mg/ml	• ΔUA-GlcNAc (Delta Di-HA) • ΔUA-2S-GalNAc-6S (Delta Di-disD) • HA 1600000 da 2.5 mg/ml • HA 190000 da 2.5 mg/ml	• ΔUA-2S-GalNAc-6S (Delta Di-disD) • ΔUA-2S-GalNAc-6S (Delta Di-disD) • ΔUA-2S-GalNAc-4S-6S (Delta Di-tisS)
Complex type N-glycans		
• Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc	• Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc	-
Sialylated glycans		
• Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc • Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc • (Neu5Acα2-8Neu5Ac)n (n<50)	• Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc • Neu5Acα2-3Galβ1-4Glc • Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc Sialyl Lewis X (S Le^{X})	-

	• Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc • (Neu5Acα2-8Neu5Ac)n (n<50)	
Ganglioside structures		
• Fucα1-2Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc (GM1a ganglioside sugar) • GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glc GD2 gangliosifde sugar	• Fucα1-2Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc (GM1a ganglioside sugar) • GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glc GT2 ganglioside sugar • GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glc GD2 gangliosifde sugar	-

Table S2. Glycans bound by *Vibrio cholerae* strains (*V. cholerae* A1552 wildtype, Rugose variant, VPS mutant (RΔ*vps-I*Δ*vps-II*)), and LPS mutant (RΔ*gmd*)) in glycan array analysis. The table shows result of glycan-binding by whole-cell *V. cholerae* strains. Glycans are clustered into classes based on their respective terminal sugars. The full data set of glycan binding is shown in Table 3. *Fuc-fucose, Gal-galactose, GalNAc 2'-N-acetyl galactosamine, Glc-glucose, GlcNAc2'-N-acetyl glucosamine, Neu5Acα2 Sialylated.

Smooth WT	**Rugose**	**VPS mutant**	**LPS mutant**
MONOSACCHARIDES & DISACCHARIDES			
• Fucα-sp3 • Glcα-sp3 • GlcNAcβ-sp3 • Rhaα-sp3	• Rhaα-sp3 • Galα-sp3 • Fucα-sp3 • Glcα-sp3	-	-

• Manα-sp3 • 6-H2PO3Manα-sp3 • 3-O-Su-Galβ-sp3 • 3-O-Su-GlcNAcβ-sp3 • 3-O-Su-GalNAcα-sp3 • Neu5Gcα-sp3 • GlcNAcβ-sp4 • 6-H2PO3Glcβ-sp4 • Neu5Acα-sp9 • GlcN(Gc)β-sp4 • 9-NAc-Neu5Acα-sp3 • Galβ1-3GlcNaAcβ-sp3 (Le^{c}) • Galβ1-3GalNAcβ-sp3 • Galα1-3GlcNAcβ-sp3 • Galβ1-3Galβ-sp3 • GalNAcβ1-3Galβ-sp3 • GalNAcα1-3Galβ-sp3 • GlcNAcβ1-4GlcNAcβ-Asn (chitobiose-Asn) • Galβ1-3GalNAcα-sp3 • Galα1-3GalNAcα-sp3 • Galα1-3Galβ-sp3 • Galβ1-2Galβ-sp3	• Manα-sp3 • 9-NAc-Neu5Acα-sp3 • 3-O-Su-GalNAcα-sp3 • 6-H2PO3Manα-sp3 • 3-O-Su-Galβ-sp3 • GlcN(Gc)β-sp4 • GlcNAcβ-sp4 • Neu5Gcα-sp3 • Neu5Acα-sp9 • Galβ1-3GlcNaAcβ-sp3 (Le^{c}) • Neu5Gcα2-6GalNAcα-sp3 • GlcNAcβ1-4GlcNAcβ-Asn (chitobiose-Asn) • Galα1-6Glcβ-sp4 • Galα1-4Galβ1-4GlcNAc-sp2 • Galα1-3GalNAcα-sp3 • 3-O-Su-GalNAcβ1-4(3-O-Su)-GlcNAcβ-sp3		

• Galβ1-4Galβ-sp4 • Fucα1-4GlcNAcβ-sp3 (Le[c]) • Galβ1-4(6-O-Su)GlcNAcβ-sp3 • GalNAcβ1-4(6-O-Su)GlcNAcβ-sp3 • 6-O-Su-GalNAcβ1-4GlcNAcβ-sp3 • GlcAβ1-3Galβ-sp33-O-Su-GalNAcβ1-4(3-O-Su)-GlcNAcβ-sp3 • Neu5Acα2-8Neu5Acα2-sp3 • 6-O-Su-Galβ1-3(6-O-Su)GlcNAcβ-sp2 • 4-O-Su-Galβ1-4GlcNAcβ-sp3 • 3,4-O-Su_2-Galβ1-4GlcNAcβ-sp3 • 6-O-Su-Galβ1-3GalNAcα-sp3 • 4,6-O-Su_2-Galβ1-4GlcNAcβ-sp2 • 6-O-Su-Galβ1-4Glcβ-sp2 • 3-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp2 • Neu5Gcα2-6GalNAcα-sp3 • Neu5Acα2-3GalNAcα-sp3	• Galβ1-4GlcNAcβ-sp3 • Galβ1-3GalNAcα-sp3 • Fucα1-3GlcNAcβ-sp3 • Galβ1-3Galβ-sp3 • GalNAcβ1-3Galβ-sp3 • GalNAcα1-3Galβ-sp3 • Neu5Acα2-8Neu5Acα2-sp3 • 6-O-Su-Galβ1-3(6-O-Su)GlcNAcβ-sp2 • 4-O-Su-Galβ1-4GlcNAcβ-sp3 • 3,4-O-Su_2-Galβ1-4GlcNAcβ-sp3 • 6-O-Su-Galβ1-3GalNAcα-sp3 • 4,6-O-Su_2-Galβ1-4GlcNAcβ-sp2 • 6-O-Su-Galβ1-4Glcβ-sp2 • 3-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp2		

• 6-O-Su-GalNAcβ1-4-(3-O-Su)GlcNAcβ-sp3 • GlcNAcβ1-4(6-O-Su)GlcNAcβ-sp2 (6-O-Su-chitobiose) • Galβ1-4(6-O-Su)Glcβ-sp2 • GlcAβ1-3GlcNAcβ-sp3 • 6-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp2 • 4,6-O-Su_2-GalNAcβ1-4GlcNAcβ-sp3 • 3-O-Su-Galβ1-4(6-O-Su)Glcβ-sp2	• Neu5Gcα2-6GalNAcα-sp3 • Neu5Acα2-3GalNAcα-sp3 • 6-O-Su-GalNAcβ1-4-(3-O-Su)GlcNAcβ-sp3 • GlcNAcβ1-4(6-O-Su)GlcNAcβ-sp2 (6-O-Su-chitobiose) • 6-O-Su-GalNAcβ1-4-(3-O-Su)GlcNAcβ-sp3 • Galβ1-4(6-O-Su)Glcβ-sp2 • GlcAβ1-3GlcNAcβ-sp3 • 6-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp2 • 4,6-O-Su_2-GalNAcβ1-4GlcNAcβ-sp3		
TRISACCHARIDES & TETRASACCHARIDES			
• GlcNAcβ1-4Galβ1-4GlcNAcβ-sp2 • Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ-sp3 • Neu5Acα2-3Galβ1-3GalNAcα-sp3	• GlcNAcβ1-4Galβ1-4GlcNAcβ-sp2 • Fucα1-2(GalNAcα1-3)Galβ-sp3	-	-

• Neu5Acα2-3Galβ1-4GlcNAcβ-sp3 • Galα1-3(Neu5Acα2-6)GalNAcα-sp3 • Galβ1-3Galβ1-4GlcNAcβ-sp4 • Neu5Acα2-6Galβ1-3GlcNAc-sp3 • GlcNAcβ1-3(GlcNAcβ1-6)GalNAcα-sp3 • Neu5Gcα2-3Galβ1-3GlcNAcβ-sp3 • 3-O-Su-Galβ1-3(Fucα1-4)GlcNAcβ-sp3 (Su-Le[a]) • Fucα1-2(Galα1-3)Galβ-sp3 • Neu5Acα2-3-(6-O-Su)Galβ1-4GlcNAcβ-sp3 • GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2 • Neu5Gcα2-3Galβ1-4GlcNAcβ-sp3 • GlcNAcβ1-2Galβ1-3GalNAcα-sp3 • Neu5Acα2-6Galβ1-4Glcβ-sp2 • Neu5Acα2-3Galβ1-3-(6-O-Su)GalNAcβ-sp3 • GlcNAcβ1-3Galβ1-3GalNAcα-sp3	• GlcNAcβ1-3Galβ1-4Glcβ-sp2 • Galβ1-2Galα1-4GlcNAcβ-sp4 • Galβ1-3(Fucα1-4)GlcNAcβ-sp3 (Le[a]) • Neu5Acα2-6Galβ1-3(6-O-Su)GlcNAc-sp3 • GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 • Neu5Acα2-3Galβ1-4Glcβ-sp3 • Neu5Acα2-3Galβ1-4GlcNAcβ-sp3 • GalNAcβ1-4Galβ1-4Glcβ-sp3 • Galα1-3(Neu5Acα2-6)GalNAcα-sp3 • GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 • Galβ1-3Galβ1-4GlcNAcβ-sp4 • Neu5Acα2-3Galβ1-4-(6-O-Su)GlcNAcβ-sp3		

• Neu5Acα2-3Galβ1-4Glcβ-sp4 • 9-NAc-Neu5Acα2-6Galβ1-4GlcNAcβ-sp3 • GlcNAcβ1-3Galβ1-4Glcβ-sp2 • Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ-sp3 • Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAcα-sp3 • 3-O-SuGalβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 • Neu5Acα2-3(GalNAcβ1-4)Galβ1-4Glcβ-sp2 (GM2) • Fucα1-2(GalNAcα1-3)Galβ1-3GalNAcα-sp3 (Blood Group A (type 3) • Fucα1-2(Galα1-3)Galβ1-4GlcNAcβ-sp3 Blood Group B (type 2) • Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-sp3 Asialo-GM1 • Fucα1-3(Neu5Acα2-3Galβ1-4)6-O-Su-GlcNAcβ-sp3 • Galβ1-3GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2	• Neu5Acα2-6Galβ1-3GlcNAc-sp3 • GlcNAcβ1-3(GlcNAcβ1-6)GalNAcα-sp3 • Galβ1-4GlcNAcβ1-6GalNAcα-sp3 • Neu5Gcα2-6Galβ1-4GlcNAcβ-sp3 • Neu5Gcα2-3Galβ1-3GlcNAcβ-sp3 • Fucα1-3(Galβ1-4)GlcNAcβ-sp3 (Le^x) • Galβ1-3GalNAcβ1-3Gal-sp4 • Galβ1-4GlcNAcβ1-3GalNAcα-sp3 • Neu5Acα2-6Galβ1-3(6-O-Su)GlcNAc-sp3 • Neu5Acα2-3Galβ1-3-(6-O-Su)GalNAcβ-sp3 • Neu5Gcα2-3Galβ1-4GlcNAcβ-sp3 • Neu5Acα2-6Galβ1-4-(6-O-		

• Galβ1-3GlcNAcα1-3Galβ1-4GlcNAcβ-sp3 • Fucα1-2(GalNAcα1-3)Galβ1-4GlcNAcβ-sp3 Blood Group A (type 2) Fucα1-3(Fucα1-2Galβ1-4)GlcNAcβ-sp3 (Le^y)	Su)GlcNAcβ-sp3 • GlcNAcβ1-2Galβ1-3GalNAcα-sp3 • Galβ1-4GlcNAcβ1-6GalNAcα-sp3 • 9-NAc-Neu5Acα2-6Galβ1-4GlcNAcβ-sp3 • GlcNAcβ1-3Galβ1-4Glcβ-sp2 • Neu5Acα2-6Galβ1-4Glcβ-sp2 • Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ-sp3 • Galβ1-3GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2 • 4-O-SuGalβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 • Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAcα-sp3 • Galα1-3Galβ1-4GlcNAcβ1-3Galβ-sp3		

	• Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 • Galβ1-3GlcNAcα1-3Galβ1-4GlcNAcβ-sp3 • Fucα1-2(Galα1-3)Galβ1-3GalNAcβ-sp3 Blood Group B (type 4) • Fucα1-2(Galα1-3)Galβ1-3GlcNAcβ-sp3 Blood Group B (type 1) • Galβ1-3GlcNAcβ1-3Galβ1-3GlcNAcβ-sp3 (Le^c) • Neu5Acα2-3(GalNAcβ1-4)Galβ1-4Glcβ-sp2(GM2) • Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-sp3 Asialo-GM1 • Fucα1-2(GalNAcα1-3)Galβ1-3GalNAcα-sp3		

	Blood Group A (type 3) • Galβ1-3GlcNAcα1-6Galβ1-4GlcNAcβ-sp2 • Fucα1-3(Fucα1-2Galβ1-4)GlcNAcβ-sp3(Le^y) • Fucα1-3(Neu5Acα2-3(6-O-Su)Galβ1-4)GlcNAcβ-sp3 • Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAcα-sp3 • (Glcα1-4)$_4$β-sp4 • Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2 • GalNAcβ1-3Galα1-4Galβ1-4Glcβ-sp3		
PENTA-NONA SACCHARIDES			
• Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ-sp4 • Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc-sp2	• Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp4 • Galα1-3Galβ1-4GlcNAcβ1-	--	-

• Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc-sp2 • GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glc-sp2 GD2 • (Galβ1-4GlcNAcβ1-3)3-sp3 • Lex1-6'(6'SLN1-3')Lac-sp4 • GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAcβ-sp2 • (A-GN-M)2-3,6-M-GN-GNβ-sp4 • Lex1-6'(Leb1-3')Lac-sp4 TFLNH • (Glcα1-6)$_6$β-sp4 • Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ-sp4 • (GN-M)2-3,6-M-GN-GNβ-sp4 •	3Galβ1-4Glcβ-sp4 • Fucα1-3(Neu5Acα2-3 Galβ1-4)GlcNAcβ1-3Galβ-sp3 • GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glc-sp2 GD2 • (Glcα1-6)6β-sp4 • Lex1-6'(Leb1-3')Lac-sp4 TFLNH • (A-GN-M)2-3,6-M-GN-GNβ-sp4 • Fucα1-3(Fucα1-2Galβ1-4)GlcNAcβ1-3Galβ1-4Glcβ-sp4 (Le^y-Lac) • (Neu5Acα2-8)2Neu5Acα2-3(GalNAcβ1-4)Galβ1-4Glc-sp2 GT2 • Fucα1-2Galβ1-3GlcNAc Blood Group H Type II Trisaccharide • Fucα1-2Galβ1-3GlcNAcβ1-		

	3Galβ1-4Glcβ-sp4 Blood Group H type1Lac • Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp2 Blood Group H (type1) penta • (Galβ1-4GlcNAcβ1-3)3-sp3 • Lex1-6'(6'SLN1-3')Lac-sp4 • Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 • Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc-sp2 • Lex1-6'(Led1-3')Lac-sp4 • (Glcα1-6)5β-sp4 • Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ-sp4		
Terminal Galactose			
• Galβ1-4Gal	• Galβ1-4Gal	• Galβ1-4Gal	-

• Galβ1-3GlcNAc • Galα1-4Galβ1-4Glc • Galβ1-3(Fucα1-2)Gal Blood Group B Trisaccharide • Galα1-3Galβ1-4Galα1-3Gal • Galβ1-3GalNAcβ1-4Galβ1-4Glc asialo GM1 • Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	• Galβ1-3GlcNAc • Galα1-4Galβ1-4Glc • Galβ1-3GlcNAcβ1-3Galβ1-4Glc • Galβ1-3GalNAcβ1-4Galβ1-4Glc asialo GM1 • Galα1-4Galβ1-4GlcNAc P1 antigen • Galα1-3(Fucα1-2)Galβ1-4(Fucα1-3)Glc • Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc • Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	• Galβ1-6Gal • GalNAcβ1-3Gal • Galα1-4Galβ1-4Glc • Galβ1-3GalNAcα1-O-Ser • GalNAcβ1-4Gal • Galα1-4Galβ1-4GlcNAc P1 antigen • Galβ1-3(Fucα1-2)Gal	
Fucosylated glycans			
• Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc • Fucα1-2Galβ1-4(Fucα1-3)GlcNAc LewisY (Le^{Y}) • Fucα1-2Galβ1-4Glc • Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-	• Fucα1-2Galβ1-4(Fucα1-3)GlcNAc LewisY (Le^{Y}) • Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc • Galβ1-4(Fucα1-3)Glc	• Fucα1-2Galβ1-4(Fucα1-3)GlcNAc LewisY (Le^{Y}) • Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc • Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc	• Fucα1-2Galβ1-4(Fucα1-3)GlcNAc LewisY (Le^{Y}) • SO3-3Galβ1-3(Fucα1-4)GlcNAc Sulpho

3)GlcNAcβ1-3Galβ1-4Glc • Galβ1-4(Fucα1-3)GlcNAc • Galβ1-3(Fucα1-4)GlcNAc Lewis A (Le^{a}) • Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc • Galβ1-4(Fucα1-3)Glc Galβ1-4(Fucα1-3)GlcNAcβ1-3Gal (Le^{x}) • GalNAcα1-3(Fucα1-2)Gal Blood Group A trisaccharide • Galβ1-4(Fucα1-3)GlcNAcβ1-3Gal • Galα1-3(Fucα1-2)Galβ1-4(Fucα1-3)Glc Blood Group B pentasaccharide •	• Fucα1-2Galβ1-4Glc • Galβ1-4(Fucα1-3)GlcNAcβ1-3Gal (Le^{x}) • GalNAcα1-3(Fucα1-2)Gal Blood Group A trisaccharide • Galβ1-3(Fucα1-4)GlcNAc Lewis A (Le^{a}) • Fucα1-2Galβ1-3GalNAcβ1-3Gal Blood group H antigen tetraose type 4 • Galβ1-4(Fucα1-3)GlcNAc • Fucα1-2Galβ1-3(Fucα1-4)GlcNAc Lewis B (Le^{b}) tetrasaccharide • GalNAcα1-3(Fucα1-2)Galβ1-4(Fucα1-3)Glc Blood group A pentasaccharide • Galα1-3(Fucα1-2)Galβ1-4(Fucα1-3)Glc Blood Group B	• Fucα1-2Gal • Fucα1-2Galβ1-4Glc • Galβ1-4(Fucα1-3)Glc • Galβ1-4(Fucα1-3)GlcNAc • Fucα1-2Galβ1-3GalNAcβ1-3Gal • Fucα1-2Galβ1-4(Fucα1-3)GlcNAc • Fucα1-2Galβ1-3GlcNAc Blood group H antigen • GalNAcα1-3(Fucα1-2)Galβ1-4(Fucα1-3)Glc • Fucα1-2Galβ1-3(Fucα1-4)GlcNAc • SO3-3Galβ1-3(Fucα1-4)GlcNAc Sulpho Lewis A (Le^{a}) • Galα1-3(Fucα1-2)Galβ1-4(Fucα1-3)Glc • GalNAcα1-3(Fucα1-2)Galβ1-3GalNAcβ1-3Gal • Galα1-3(Fucα1-2)Galβ1-3GalNAcβ1-3Gal	Lewis A (Le^{a})

	pentasaccharide		
Mannosyl containing glycans			
• Manα1-2Man	• Manα1-3(Manα1-6)Manβ-sp4 • Manα1-2Man • Manα1-6Man	• Manα1-3Man • Manα1-6Man	• Manα1-6Man
Glycosaminoglycans - high and low molecular weight			
• ΔUA-GlcNAc (Delta Di-HA) • HA-16 4.9mM • ((±2S)GlcA/IdoAα/b 1-3(±4S)GalNAcβ1-4)n (n<250) • HA - 4 10mM • HA 190000 da 2.5 mg/ml	• ΔUA-GlcNAc (Delta Di-HA) • ΔUA-2S-GalNAc-6S (Delta Di-disD) • HA 1600000 da 2.5 mg/ml • HA 190000 da 2.5 mg/ml	• Neocarratetraose-41-O-sulphate (Na+) • ΔUA-2S-GalNAc-6S (Delta Di-disD) • ΔUA-2S-GalNAc-6S (Delta Di-UA2S) • ΔUA-2S-GalNAc-4S-6S (Delta Di-tisS) • ΔUA-2S-GlcNS-6S • ((±2S)GlcA/IdoAα/b 1-3(±4S)GalNAcβ1-4)n (n<250)	• ΔUA-2S-GalNAc-4S-6S (Delta Di-tisS) • ΔUA-2S-GalNAc-6S (Delta Di-UA2S) • ΔUA-2S-GalNAc-6S (Delta Di-disD)
Complex type N-glycans			
• Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc	• GlcNAcβ1-4GlcNAcβ1-4GlcNAc • Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc	• GlcNAcβ1-4GlcNAcβ1-4GlcNAc • GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAc	-
Sialylated glycans			

• Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc • Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc • (Neu5Acα2-8Neu5Ac)n (n<50)	• Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc • Neu5Acα2-3Galβ1-4Glc • Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc Sialyl Lewis X (S Le^X) • Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc • (Neu5Acα2-8Neu5Ac)n (n<50)	• Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc • Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc • Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc • Galβ1-4(Fucα1-3)GlcNAcβ1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-3)Galβ1-4Glc • Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc	• Galβ1-4(Fucα1-3)GlcNAcβ1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-3)Galβ1-4Glc
Ganglioside structures			
• Fucα1-2Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc (GM1a ganglioside sugar) • GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glc GD2 gangliosifde sugar	• Fucα1-2Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc (GM1a ganglioside sugar) • GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glc GT2	• Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-8 Neu5Acα2-3)Galβ1-4Glc GT1c ganglioside sugar • Neu5Acα2-8Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc GT1a ganglioside sugar	• Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-8 Neu5Acα2-3)Galβ1-4Glc

	ganglioside sugar • GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glc GD2 gangliosifde sugar	• Fucα1-2Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc (GM1a ganglioside sugar)	