Resilient *Hymenobacter* strains isolated from Icelandic environments
Exploring osmo-tolerance and interspecific interactions

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Resilient *Hymenobacter* strains isolated from Icelandic environments
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Abstract

Microorganisms are constantly exposed to various kinds of stress in their natural environments. The mechanisms for adaptation to these stressors reflect their life strategies, determining to some extent their distribution in the environment. Bacteria live in dense communities characterised by complex interspecific interactions. Studying bacterial interactions and their competitive strategies can further explain their environmental distribution. Members of the genus *Hymenobacter* are often characterised with respect to their cold and radiation tolerance, but little is known about their tolerance of low water activity or their interspecific interactions. The tolerance of *Hymenobacter* strains H16F320, JF1031 and LW0504 to hyperosmotic shock was investigated by lowering the water activity of the culture medium with various humectants, in order to account for specific solute effects. The strains exhibited a resounding and statistically significant inability to tolerate the conditions imposed on them, which raises questions about the strategies they employ to cope in their harsh natural environments. The interspecific interactions of H16F320 and LW0504 were also assessed by culturing them with various co-isolated strains in an interaction assay. The presence of other strains unanimously reduced growth of the *Hymenobacter* strains, suggesting their competitive incompetence. In many of the co-cultures, a twitching motility phenotype was induced in the *Hymenobacter* strains, which were previously shown to be nonmotile in monoculture, revealing the benefit of studying bacteria as members of a larger community. The nutritional and physical environment of the cultures influenced this induced motile phenotype: nutrient deprivation consistently reduced the extent of twitching motility, while low agar concentrations completely inhibited it. The strains did not initiate biofilm formation when cultured individually in polystyrene microtiter wells. Further study on interspecific interactions and stress tolerance will increase our understanding of the functional and ecological role of the *Hymenobacter* isolates in the natural communities they inhabit.

**KEY WORDS:** Osmotic stress; water activity; co-culture; competition; surface motility.
Ágrip


LYKILORÐ: Osmósu-stress; vatnsvirkni; samrækt; samkeppni; yfirborðskvikleiki.
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1 Introduction

1.1 Hymenobacter

1.1.1 Hymenobacter taxonomy

The genus *Hymenobacter* was first described by Hirsch *et al.* (1998) when they isolated a novel bacterium from soil in the Dry Valleys region of Antarctica and subsequently assigned it with the name *Hymenobacter roseosalivarius*, which consequently became the type species of the genus. In 2006, the genus was amended by Buczolits *et al.*; this included reclassification of three non-valid ‘Taxeobacter’ species, previously described by Reichenbach (1992), as members of the genus *Hymenobacter*. The genus was amended again by Han *et al.* in 2014. *Hymenobacter* represents a deep branch within the *Bacteroidetes* phylum, which is exhibited by the uncharacteristically high G+C content (55-70 mol%) of the genomic DNA in members of the genus (Buczolits *et al.*, 2002; Liang *et al.*, 2019).

Originally classified within the phylum *Bacteroidetes*, order *Sphingobacteriales* and family *Cytophagaceae*, the genus has recently been placed in the new family *Hymenobacteraceae*, along with *Adhaeribacter*, *Nibribacter*, *Pontibacter* and *Rufibacter* (Munoz, Rosselló-Móra and Amann, 2016). In the last two decades, numerous novel species in the *Cytophaga-Flavobacterium-Bacteroides* group have been discovered, resulting in rapid changes in the taxonomy of the phylum *Bacteroidetes* (Sedláček *et al.*, 2019). Due to phylogenetic clustering of *Hymenobacter* spp., the creation of two new genera, *Siccationidurans* and *Parahymenobacter*, has been proposed, but these names have yet to be validated (Reddy, 2013). At the present time, the genus *Hymenobacter* consists of 80 species with validly published names (Euzéby, n.d.), many of which have been described recently (Han *et al.*, 2018; Liang *et al.*, 2019; Nie *et al.*, 2019; Ten *et al.*, 2019; Feng *et al.*, 2019; Sedláček *et al.*, 2019).
1.1.2 Genus description

Members of the genus *Hymenobacter* produce cells that are Gram-stain-negative, rod-shaped and form polyphosphate granules near the cell poles. Cells are brick-red, red-pink or pink pigmented with many shades of colours, and they spread in thin layers on agar surfaces (Buczolits and Busse, 2015; Klassen and Foght, 2008). Strains are typically non-motile, and this is commonly regarded as characteristic of the genus; however, members of the former genus ‘*Taxeobacter*’ were described as exhibiting gliding motility, suggesting that at least some species within the *Hymenobacter* genus have this ability (McBride et al., 2014). The fatty acid profile of *Hymenobacter* species includes predominantly branched fatty acids including major acids iso-C15:0, anteiso-C15:0, summed feature 3 (C16:1 ω7c and/or C16:1 ω6c), and summed feature 4 (C17:1 iso I and/or C17:1 anteiso B)c. Members of the genus exhibit a polar lipid profile with phosphatidylethanolamine as the main component and a unique, complex mixture of several other lipids. The principal menaquinone is MK-7 (Buczolits and Busse, 2015; Buczolits et al., 2006).

*Hymenobacter* sp. are metabolically heterotrophic with a preference for oligotrophic environments, growing aerobically with or without light. Strains are catalase-positive, but oxidase activity varies. Typical growth parameters include a temperature range between 4-37°C, and an optimum between 10-28°C; generally, neutral pH and low salt concentrations are preferred. The carbon utilisation spectrum is reportedly fairly limited, including some sugars, sugar alcohols, organic acids, and a few amino acids: hydrolysis of gelatin, starch, xylan, Tween 80 and Tween 60 is common (Buczolits and Busse, 2015). Strains within the genus are commonly reported to exhibit metabolic capabilities pertaining to their resistance to oxidative stress, including production of large amounts of extracellular polymeric substance (EPS) and synthesis of unique UVR-protective 2-hydroxy-carotenoid pigments (Koo et al., 2014; Baker et al., 2010; Klassen and Foght, 2008). From the limited studies on *Hymenobacter* carotenoid pigment production, it has been proposed that members of the genus have undergone a complex non-vertical evolution resulting in a difficulty in assigning novel isolates to described species. Klassen and Foght (2011) suggest that this could be due to a propensity for horizontal gene transfer as well as preservation of various genotypes in ice-bound dormancy, allowing for the co-existence of ancient and modern genotypes and ultimately facilitating a web-like, instead of the traditional tree-like, pattern of evolution.
1.1.3 Ecological diversity

Species of the genus *Hymenobacter* are commonly isolated from oligotrophic environments, and have been found to occupy a variety of ecological niches, including soil (Hoang et al., 2013; Ohn et al., 2018; Kim, Im and Lee, 2008; Srinivasan et al., 2015), arid lands (Reddy and Garcia-Pichel, 2013), wetland freshwater (Baik et al., 2006), lakes (Kang et al., 2018), and deserts (Zhang et al., 2009). They have also been isolated as airborne bacteria (Buczolits et al. 2002), in a uranium mine waste water treatment system (Chung et al., 2010), acid water neutralization facility waste water (Kang et al., 2018), abandoned arsenic-contaminated farmland soil (Nie et al., 2019) and in abandoned lead-zinc ore (Feng et al., 2019). Members of *Hymenobacter* are often associated with extreme and often cold environments including Antarctic soils, lakes and rocks (Oh et al., 2016; Koo et al., 2014; Hirsch et al., 1998; Jiang et al., 2018; Sedláček et al., 2017), glaciers and glacial till (Liu et al., 2016; Klassen and Foght, 2011; Chang et al., 2014; Gu et al., 2017), and permafrost regions (Zhang et al., 2007; Han et al., 2014). Commonly, they exhibit a considerable tolerance to radiation (Jung et al., 2014; Kim et al., 2016; Lee et al., 2017); representatives of the genus have been isolated from irradiated pork (Collins et al., 2000), and have repeatedly been found to be one of the dominant genera on solar panels (Porcar et al., 2018; Dorado-Morales et al., 2016; Tanner et al., 2017).

The environments from which *Hymenobacter* species have been isolated are clearly often characterised by low temperatures and UV irradiation, but these extreme niches also experience frequent fluctuations in other conditions including freeze-thaw cycles, dehydration, osmotic stress and low nutrient concentrations (Peeters et al., 2011). Despite exposure to such abiotic stressors, the snow and ice surface environment of polar and other cold regions thrive with microbial life. Psychrophilic algal blooms are observed seasonally with green, orange and red coloration on the snow and ice surface (Terashima et al., 2018). Snow algae are widespread in alpine regions and on icefields, having been described in North America, Greenland, Iceland, Europe, the Russian Arctic, the Himalaya, Japan, South America and Antarctica (Havig and Hamilton, 2019). The algae utilise light energy for photosynthesis, but interestingly, non-photosynthetic heterotrophic microorganisms are commonly found to thrive in these same environments. Accordingly, *Hymenobacter* isolates have often been isolated from areas characterised by red-snow algae in polar regions (Havig and Hamilton, 2019; Terashima et al., 2018; Sedláček et al., 2017; Kojima et al., 2016; Fuji et al., 2010). Similarly, *Hymenobacter*
species are among the non-phototrophic microbiota commonly associated with lichens in polar regions (Sigurbjörnsdóttir et al., 2014; Lee et al., 2014; Kim, Park and Oh, 2014; Ahn et al., 2014; Oh et al., 2016). Lichens, the symbiotic associations of fungi and photoautotrophs (algae and/or cyanobacteria), are capable of persisting under extreme ecological conditions owing to their physiological integration of organisms, and can survive for thousands of years: thus, they represent a fairly stable ecological niche for other microorganisms (Cardinale et al., 2008). Indeed, recent culture-independent studies have revealed the diversity and abundance of non-phototrophic lichen-associated bacteria, resulting in many researchers postulating their potential roles in the symbiosis (Grube et al., 2009; Hodkinson and Lutzoni, 2009; Biosca et al., 2016). For instance, phosphate-solubilization is a feature commonly observed in lichen-associated bacteria, supporting the notion that selective endothallic communities play key functional roles in lichen symbiotic associations (Liba et al., 2006; Sigurbjörnsdóttir, Andrésson and Vilhelmsson, 2015). The prevalence of *Hymenobacter* representatives in the extreme environments from which they are isolated can be ascribed to their cold and UV-radiation tolerance, as multiple studies have shown. However, less frequently studied is their potential tolerance to other abiotic factors such as osmotic stress and dehydration, as well as biotic factors such as interspecific competition. Research into these variables could further elaborate on the ecological role of members of the genus *Hymenobacter*.

1.2 Osmoregulation

1.2.1 Osmotic stress

Among the many abiotic encumbrances imposed on organisms adapted to life in cold environments is osmotic stress. In order to grow, bacterial cells require a precise balance in the flow of water in and out of the cell. While essentially a passive process owing to the high permeability of bacterial membranes to water, the flow of water is nonetheless tightly regulated in order to facilitate growth. As the mass of macromolecules (DNA, proteins, lipids) increase with the growth of the cell, the cell itself must also expand in order to be able to contain these molecules; the flow of water into the cell allows for this expansion by generating an outward mechanical force (O’Byrne and Booth, 2002). In order to maintain this outward turgor pressure on the cell wall, the internal osmotic pressure of the microbial cell must exceed that of the surrounding medium (Tapia, Alzamora and Chirife, 2007). To accomplish this,
bacteria accumulate solutes in their cytoplasm to concentrations far greater than their metabolic needs would dictate, solely to maintain a higher osmotic pressure than outside the cell (O’Byrne and Booth, 2002).

Temperatures below freezing point, drought, and high substrate salinity are common conditions in cold environments, all of which have the shared consequence of water withdrawal from the cell (Tartari and Forlani, 2008). This is because of the increased concentration of solute in the environment; in order to counteract this, water flows out of the cell until the osmotic pressure of the cytoplasm is re-stabilised relative to the environment (O’Byrne and Booth, 2002). Cold ecosystems are often characterised by fluctuations in environmental conditions, such as frequent freeze-thaw cycles (Collins and Margesin, 2019); as such, osmotic stress is a consistent complication that bacteria must contend with. When faced with a sudden external change in osmolarity, cells are faced with hyperosmotic shock, rapidly losing water in a process called plasmolysis (Sperber, 1983). Koujima et al. (1978) showed that when the concentration of NaCl in the medium is increased from 0,1M to 1,8M (corresponding to a decrease in $a_w$ from 0,995 to about 0,950), Staphylococcus aureus rapidly loses about 50% of its water. In the absence of a counteractive response, this effect is lethal, having deleterious consequences on the cell’s DNA, proteins and cell membrane (Ramos et al., 2001). For instance, the dehydration of the cell membrane causes an increase in the transition temperature – that is, the membrane shifts to a gel phase at temperatures where it would normally be in a liquid crystalline phase (Halverson and Firestone, 2000), thus reducing membrane fluidity. The same also applies vice versa, when for instance ice thaws resulting in hypoosmotic shock whereupon water flows along the osmotic gradient into the cell, which causes it to swell and potentially burst (Kempf and Bremer, 1998).

1.2.2 Compatible solutes

During plasmolysis, cell growth is interrupted (Sperber, 1983), and the organism will either die or remain in the lag-phase until the membrane turgor it requires for growth is re-established. In order to regain its intracellular osmotic pressure, the cell must lower its water activity until it is equal to or slightly lower than that of the surrounding medium (Tapia et al., 2007). This immediate adaptive response to hyperosmotic shock primarily involves the accumulation of solutes in the cytoplasm, and is essentially a universal reaction to high extracellular osmolarity,
since the physicochemical effects of osmotic shock are the same for all cells (Csonka and Hanson, 1991). Osmoregulation, therefore, refers to the precise control over the influx and efflux of solutes and the concomitant passive movement of water to balance and maintain turgor pressure (O’Byrne and Booth, 2002). However, in order for the cell to avoid metabolic disruption, its ionic composition, pH level and metabolite content must also exist in a delicate balance. Thus, the maintenance of metabolism imposes constraints on the nature of solute the cell can use to counteract osmotic shock (Csonka and Hanson, 1991). In the presence of high salt concentrations, for example, only the halophilic or halotolerant will be able to retain their activity, while the enzymes of other organisms will begin to denature (Gutierrez, Abee and Booth, 1995). To permit cell viability and growth over a range of osmotic conditions without interfering with metabolism, cells accumulate specific organic osmolytes known as compatible solutes (Breed et al., 2001). These compatible solutes are small, inert organic molecules which do not interfere with cellular functions such as DNA replication and DNA-protein interactions, even as they are amassed at high concentrations, even up to molar levels (Tapia et al., 2007; O’Byrne and Booth, 2002; Kempf and Bremer, 1998). As well as not interfering with enzymatic activity, they have also been shown to have stabilising effects on enzymes, opposing protein unfolding and promoting subunit assembly (Gutierrez et al., 1995; Bayles and Wilkinson, 2001).

Common compatible solutes include sugars such as trehalose; polyols such as glycerol and glucosylglycerol; amino acids such as proline and glutamate; quaternary amines such as glycine betaine and carnitine; and sulphate esters such as choline-O-sulphate (Kempf and Bremer, 1998). While some can only be accumulated through transport into the cell from the environment (including choline, betaine and ectoine), others can only be accessed via intracellular synthesis, such as trehalose; others still can be either synthesised or transported, as is the case for proline (O’Byrne and Booth, 2002). The reservoir of compatible solutes bacteria can utilise are specific to each species, depending on their synthetic machinery and membrane transport systems. Bayles and Wilkinson (2001) reported the activity of betaine, proline betaine, acetyl carnitine, carnitine, y-butyrobetaine and 3-dimethylsulphoniopropionate as compatible solutes for Listeria monocytogenes, while no osmoprotective activity was found with ectoine, hydroxyectoine, pipecolic acid or proline. The growth of E. coli under hyperosmotic conditions is facilitated by its concomitant synthesis of trehalose in the absence of other compatible solutes in the growth medium (Larsen et al.,
Prominent compatible solutes utilised by *Staphylococcus aureus* include proline and glycine betaine, while taurine, proline betaine and carnitine have also been shown to act as osmoprotectants (Vilhelmsson and Miller, 2002).

### 1.2.3 Specific solute effects

As previously mentioned, a reduction in water activity has a dramatic effect on bacterial growth, including an increase in the lag phase and decreases in the growth rate as water activity is reduced below the optimum, until it reaches the minimum $a_w$ for growth at which point the cell will either remain dormant or die (Sperber, 1983). A good example of these parameters can be described in the form of *S. aureus*, where the optimum $a_w$ for the growth of 14 food-poisoning strains was determined to be between $a_w$ 0.995 and 0.990; as the $a_w$ was reduced from 0.99 to 0.90 there was an almost linear reduction in the growth rate. At $a_w$ 0.90 the strains exhibited a growth rate which was only 10% of the maximum rate, and the lowest $a_w$ facilitating observable growth was 0.86 (Scott, 1957). The availability of compatible solutes in the environment has a pronounced effect on the growth of organisms under hyperosmotic stress (Tapia *et al.*, 2007). For example, Bayles and Wilkinson (2001) demonstrated that compatible solutes increased the growth rate of NaCl-stressed *L. monocytogenes* cells up to 2.6-fold compared to similarly stressed cells without exogenous compatible solutes.

In order to study the effect of lowering $a_w$ on the bacterial response, many studies use solutes commonly referred to as humectants to lower the water activity of the medium (Ballesteros *et al.*, 1993; Santos *et al.*, 1994; Marshall *et al.*, 1971; Stewart *et al.*, 2004; Cebrián *et al.*, 2015; Wdowiak-Wróbel *et al.*, 2016). To further complicate things, the nature of the humectant used can also impact the cell’s response, meaning that different $a_w$-lowering solutes will provoke different osmoregulatory reactions, termed ‘specific solute effects’ (Chirife, 1994). For instance, at concentrations conferring the same $a_w$ values, glycerol has been found to be less inhibitory than NaCl for many pathogenic bacteria, including *C. botulinum*, *C. perfringens*, *V. parahaemolyticus*, *B. cereus*, *L. monocytogenes*, and *E. coli* (Tapia *et al.*, 2007). In a study by Marshall *et al.* (1971), glycerol was postulated to be less inhibitory than sodium chloride to salt-sensitive species of bacteria; conversely, salt-tolerant bacteria exhibited the opposite trend, with glycerol generally being more inhibitory than salt. The authors also speculated that cell morphology could be in some way linked to the bacterial tolerance to the two different humectants, observing that glycerol was much more inhibitory than NaCl when the bacteria
were cocci. Rod-shaped bacteria, in contrast, were more sensitive to NaCl (Marshall et al., 1971); however, only 16 strains were studied, and so it is difficult to derive conclusive relationships between morphology and specific solute osmotic stress tolerance.

The relative tolerance of most salt-tolerant species of bacteria to glycerol can be explained in terms of membrane permeability: glycerol can freely enter the bacterial cell, and is therefore not as big a stressor as other solutes. In general, solutes such as NaCl, KCl, sucrose and glucose exhibit relatively similar patterns while glycerol usually permits growth at lower a_w levels (Sperber, 1983). Vilhelmsson and Miller (2002) showed that S. aureus cells accumulate the compatible solutes proline and betaine when in the presence of the impermeant humectants NaCl and sucrose, while this accumulation was not observed when cells were grown in the presence of glycerol. This would suggest that S. aureus accumulates the permeant glycerol itself as a compatible solute; in fact, it has been speculated that inhibition via glycerol is exacerbated by its transport into the cell and subsequent lowering of the intracellular a_w rather than through hypertonic stress (Cebrián et al., 2015).

1.3 The general stress response
1.3.1 General stress response of E. coli
The accumulation of compatible solutes is an intrinsic and transient mechanism in bacterial cells faced with a sudden osmotic shift; in order to survive an abrupt change in external osmolality, survival mechanisms must by necessity activate within seconds (Hohmann, 2002). Thus, while compatible solute accumulation is often dramatic, the molecular mechanisms which regulate it are often quite simple, such as the lifting of a negative control - allowing for a rapid response (Csonka and Hanson, 1991). This initial adaptive phase can be described as the short-term reaction to hyperosmotic shock; however, in order for bacterial growth to be sustained, the long-term response is required in order to re-establish homeostatic mechanisms that maintain the new osmotic steady state (Csonka and Hanson, 1991). The general stress response is induced when cells stop growing and instead use their resources entirely for maintenance and survival in the face of stress in their environment, such as that of sudden high osmolarity (Hengge, 2011). The general stress response is dependent on the use of alternative sigma factors, the recognition units of RNA polymerase which determine which promoter sequence the transcriptional machinery will bind to and therefore which genes will be
expressed (Cebrián et al., 2015). Under adverse conditions, E. coli and other related gram-negative bacteria will switch from the housekeeping subunit σ^70 to the closely related σ^S (RpoS) subunit, upon which the general stress response is dependent (Hengge, 2011). Osmotic stress therefore leads to elevated levels of rpoS in the cell by increasing the rate of both transcription and translation of rpoS mRNA and by decreasing the rate of proteolysis of the σ^S subunit (Lange and Hengge-Aronis, 1994). An elevation of rpoS levels in the cell begins the general stress response which consequently confers resistance to a variety of different stresses, including heat, high osmolarity, low pH, oxidising agents and UV irradiation; upon accumulation in the cell, σ^S directly or indirectly activates over 500 genes resulting in complex cascades and feedback loops (Cebrián et al., 2015; Hengge, 2011).

The general stress response of E. coli, mediated by σ^S, is stationary-phase specific and manifests with both morphological and physiological adaptations (Lange and Hengge-Aronis, 1991). Upon entry to the stationary phase, cells transition from their normal rods to a smaller, more ovoid shape (Hengge-Aronis, 1993). At the same time, dramatic metabolic alterations take place, with as many as 19% of σ^S-dependent genes encoding metabolic enzymes and involved in central energy metabolism. A number of these genes (involved in glycolysis, fermentation, anaerobic respiration, electron transport, and the pentose phosphate pathway) exhibit positive σ^S control, while concomitant repression of genes involved in aerobic growth, cell division and protein synthesis takes place (Weber et al., 2005; Jozefczuk et al., 2010). Induction of the general stress response upon entry into the stationary phase is therefore followed by a shift away from oxidative respiration towards a more conservative fermentative energy metabolism; this has been postulated as a protective mechanism against the potential increased formation of damaging reactive oxygen species in the electron transport chain subsequent to entry into a stressful situation (Weber et al., 2005; Nyström, 2004).

Cells that activate the general stress response also experience alterations in the composition of their outer membranes. About 14% of genes recognised by σ^S in E. coli express membrane proteins involved in transport systems, suggesting that membrane traffic is to a large extent altered in stressed cells (Weber et al., 2005). Phospholipids in the cell membrane are also modified to contain cyclopropane fatty acids, the synthesis of which depends on σ^S; this feature has been shown to bestow acid resistance to E. coli cells in the stationary phase (Chang and Cronan Jr., 1999). Membrane fluidity and stability are also important determinants
of cell endurance in times of trouble. In the case of osmotic stress, the synthesis and/or uptake of compatible solutes acts to increase membrane fluidity. Solutes such as betaine, proline and trehalose can at high concentrations reduce the temperature of the liquid crystalline to gel phase transition; this counteracts the antithetical increase of the transition temperature brought about by dehydration and low temperatures (O’Byrne and Booth, 2002; Deshnium et al., 1997). Looking at other bacterial genera, *Staphylococcus aureus* has been shown to respond to increased NaCl concentrations by increasing the cardiolipin content of the cell membrane; the proportion of branched fatty acids in cardiolipin also increases (Kanemasa et al., 1972). In *Pseudomonas putida* as well as several other gram-negative species, the ratio of trans to cis unsaturated fatty acids has been found to increase under various environmental stresses, including desiccation, temperature, and heavy metal toxicity (Heipieper et al., 1996; Halverson and Firestone, 2000). Rahpeyma and Raheb (2019) demonstrated that mutagenesis of the *rpoS* gene in *Flexibacter chinesis* resulted in a decomposition of the outer membrane, suggesting that $\sigma^S$ acts as a crucial modifier of membrane composition during stressful times.

1.3.2 Alternative sigma factors in other gram-negative bacteria

The general stress response in gram-negative bacteria has been most intensively studied using the model organism *E. coli*, where the $\sigma^S$ sigma factor exists as the master regulator (Hengge, 2011). In many other Gammaproteobacteria, especially enteric bacteria, the $\sigma^S$ sigma factor response is also utilised, albeit with modifications that reflect the adaptive requirements of their environment. For example, *Vibrio cholerae* activates *rpoS* during the mucosal escape response, resulting in an upregulation of genes involved in chemotaxis and motility (Nielsen et al., 2006). Other clades of the Proteobacteria commonly possess $\sigma^S$-like sigma factors; Betaproteobacteria have an *rpoS* regulator with 50-53% identity to the $\sigma^S$ of *E. coli* (Hengge, 2011). Among the Deltaproteobacteria, *Geobacter sulfurreducens* has been shown to express a $\sigma^S$ sigma factor, while other representatives such as *Bdellovibrio* and *Desulfovibrio* do not express *rpoS*-like genes; curiously, $\sigma^S$ has not been reported in Alpha- or Epsilonproteobacteria, the members of which use evolutionarily distinct mechanisms in their stress responses (Núñez et al., 2004; Hengge, 2011; Fiebig et al., 2016).

Other gram-negative bacteria have not been investigated as thoroughly with respect to the molecular mechanisms of their general stress responses, including those of the *Cytophaga-Flexibacter-Bacteroides* (CFB) group. Interestingly, Gruber and Bryant (1997) discovered that
the primary housekeeping sigma factor of Chlorobium tepidum, a green sulphur bacterium closely related to the CFB group, is most closely related to the stationary-phase stress response rpoS sigma factors of proteobacteria (Gruber and Bryant 1997; 1998). Bacteroides fragilis has been found to express the unusual housekeeping sigma factor σABfr, which recognises promoter sequences unique to the phyla Bacteroidetes and Chlorobi. These primary σABfr sigma factors are the only known essential sigma factors that group together with the non-essential rpoS factors (Vingadassalom et al., 2005). These observations would suggest that the mechanisms governing the general stress response of members of the CFB group, including Hymenobacter, are evolutionarily distinct from the well-studied σS-mediated response of E. coli and other eubacteria. Type-3 ECF σ-factors are non-essential sigma factors that regulate the expression of genes with extracytoplasmic function, and these have been found to be especially numerous in the Bacteroidetes/Chlorobi group: Bacteroides thetaiotaomicron, for instance, possesses 48 predicted ECF sigma factors (Kill et al., 2005). Bacteroides fragilis has been shown to utilise the ECF sigma factor EcfO to protect the cell against oxidative stress (Ndamukong et al., 2013). Similarly, genes encoding the sigma factor ECF42 have been found within the phylum Bacteroidetes, and this sigma factor has been shown to confer stress tolerance and aid with biofilm formation in Pseudomonas putida (Pinto and Mascher, 2016). ECF sigma factors could therefore be a major basis for stress tolerance for members of this phylum.

1.3.3 Cross-protection

Harsh, cold environments such as those in Iceland are characterized by multiple abiotic stress factors, including freeze-thaw cycles, osmotic stress, and UV radiation; these cyclical stressors are an almost constant encumbrance to autochthonous microbes. As rpoS activity increases after exposure of cells to various different stresses, and since σS controls large regulons that equip the cell with the means of resisting these various stresses, it therefore naturally follows that exposure of cells to one stress may subsequently confer it with resistance to many different stresses, regardless of whether this tolerance is applicable to the situation at hand. This process is known as cross-tolerance or cross-protection: for example, cells under nutritive stress that enter the stationary phase become more tolerant to hydrogen peroxide, heat, ethanol, high osmolarity, and acidic pH (Rangel, 2010). E. coli responds to glucose starvation by inducing production of about 30 proteins; some of these are heat shock proteins, and as well as conferring resistance to starvation they concomitantly induce cross protection against heat.
shock, oxidative stress and osmotic stress (Jenkins et al., 1988; 1990). Of the proteins produced in response to starvation, 11, 6 and 5 of them are common to proteins induced in *E. coli* by heat shock, oxidative stress and osmotic stress respectively, the majority of which are of the Pex (post-exponential) family (Matin, 1991).

Cross-protection is demonstrably not universal among stressors; different stressors do not automatically and reversibly induce cross-protection against other stressors (Dhar et al., 2012). For instance, in *Vibrio vulnificus*, nutrient starvation alone does not result in cross-protection against other stressors. Cells that are exposed to osmotic shock, however, develop cross-protection against shifts to high temperature and to oxidative stress (Rosche et al., 2005). Furthermore, the induction of the stationary phase alone is enough to confer cross-protection to heat shock, but the *rpoS* sigma factor is required for effective cross-tolerance to oxidative stress (Rosche et al., 2005). The cross-protective response in *V. vulnificus* is clearly complex and distinct from that of *E. coli*. Other bacterial species have been shown to display diverse cross-protective effects. For example, *S. aureus* and *Streptococcus faecium* exhibit increased radiation tolerance after being subjected to osmotic stress (Lenovich, 1987). Gaucher et al. (2019) showed that a strain of *Propionibacterium freudenreichii*, after being subjected to hyperosmotic shock and performing the necessary osmotic adaptations, also displayed enhanced heat, oxidative, freeze-drying and acid stress tolerance. Bayles and Wilkinson (2001) demonstrated that osmoprotectants (compatible solutes) utilised by *L. monocytogenes* after exposure to hyperosmotic conditions also conferred chill stress resistance to the bacterium; that is, compounds that were osmoprotectants were also found to be cryoprotectants. In a different study, 3 members of the cold shock protein family (Csp) produced by *L. monocytogenes* were found to promote both cold and osmotic stress resistance. Csps act as nucleic acid chaperones, and have been implicated in the regulation of RpoS stress response proteins (Schmid et al., 2009).

### 1.3.4 Stochasticity and Anticipation

Cross-protection, the induction of multiple stress-tolerances following one specific stress, is highly effective, imparting microbes with an overall 10-fold higher survival rate than microbes lacking this ability, regardless of the kind of stress used (Andrade-Linares, Lehmann and Rillig, 2016). Since it does not require the commitment of a direct induced response, instead improving defence against fluctuations in possible future stressors, this can be a highly cost-
saving strategy (Wesener and Tietjen, 2019). Some environmental fluctuations occur essentially randomly; microorganisms constantly faced with erratic and unpredictable environmental disturbances may employ cross-protective mechanisms, but a more frequently observed reaction is one of stochasticity: that is, randomly switching between potential alternative states in order to ensure that a portion of the population is always equipped to deal with whatever is thrown at them (Mitchell et al., 2009). For instance, starved cells of *Bacillus subtilis* will respond by inducing sporulation, but this never occurs to all cells in the population; this form of bet-hedging utilises cell heterogeneity to improve the overall fitness of a genetically identical population (Jõers and Tenson, 2016; Rillig et al., 2015). Indeed, gene expression itself can be described as an inherently stochastic process, as transcription factors are generally low in number and therefore have limited availability: thus, genetically identical cells often exhibit variable responses to the same stimuli (Meriem et al., 2019).

On the other end of the spectrum, environmental fluctuations can also be highly predictable, such as periodic changes in photon flux as a result of the Earth’s rotation (Tagkopoulos, Liu and Tavazoie, 2008). Here, a third mechanism can be used by microorganisms to respond to disturbance, known as anticipation, or predictive adaptation. Cells utilising this strategy rely on a present environmental signal to predict future environmental change, and use this signal to preadapt to the future condition – like preparing your photosynthetic machinery before sunrise (Dhar et al., 2012; Tagkopoulos et al., 2008). For instance, Tagkopoulos et al. (2008) showed that *E. coli* responds to a temperature increase from ambient levels (<30°C) to 37°C by expressing the same genes as it would responding to oxygen downshift. Transitioning from the outside environment into the human oral cavity exposes *E. coli* to a temperature of 37°C, followed by an impending drop in oxygen levels as it enters the gastrointestinal tract (Dhar et al., 2012). Thus, *E. coli* uses the signal of a temperature increase to predictively adapt to future oxygen downshift, for instance by switching to anaerobic pathways. This confers strong fitness advantages as the bacterium will be in the ideal physiological state to survive the GI tract before it even enters it (Tagkopolous et al., 2008). This phenomenon is not a trivial consequence of cross-protection, but an evolved anticipatory mechanism; Tagkopolous et al. conducted a laboratory evolution experiment, introducing *E. coli* to a novel environment where the relationship between temperature and oxygen was inverted. Remarkably, the bacterium was able to decouple the sequential transcriptional
responses, since it was no longer beneficial to induce anaerobic conditions following
temperature upshift (Mitchell et al., 2009). In another evolution experiment, Dhar et al. (2012)
showed that *Saccharomyces cerevisiae*, after being cyclically subjected to salt (osmotic) and
oxidative stress every 10 generations for 300 generations, developed a predictive cross-
tolerance to salt stress when exposed to oxidative stress. Curiously, this anticipation did not
evolve vice-versa. Environmental variables hitherto have either been presented as perfectly
predictable or completely random; purely stochastic and anticipatory mechanisms describe
responses to these unrealistic extremes. In reality, parameters may seem random when viewed
in isolation but actually follow a fairly predictable pattern in the context of other
interconnected variables; furthermore, stressors can appear together or in rapid succession,
and can oscillate between states of order and entropy – that is, there can be changes in the
way they fluctuate (Tagkopolous et al., 2008; Andrade-Linares et al., 2016; Katz and Springer,
2016). In lieu of mutual exclusivity, these three general strategies can therefore be employed
simultaneously, in a complicated optimization of cost-benefit dynamics.

1.4 Microbial communities and competition
1.4.1 Costs and benefits of stress responses
The mechanisms described above as microbial reactions to various abiotic stressors are clearly
very complex, requiring the evolutionary development of sophisticated regulatory functions;
why, then, do microorganisms continuously exposed to multiple fluctuating stressors not
simply constitutively express their entire range of protective responses? Constitutive
adaptations to stress become imprinted in the genomes of species if the stress is a constant
part of the environment, such as psychrophilic tolerance to cold temperatures. Inducibility of
stress responses, conversely, is employed as a basic cost-saving strategy to more transient
stress factors; it would be a waste of resources to always be prepared for situations that rarely,
or unpredictably, arise (Hilker et al., 2016). This becomes significant when shifting from the
perspective of axenic laboratory research to the wider environmental community context; the
outcomes of stress manifest at the physiological level, as previously detailed, but also
community composition levels (Schimel, Balser and Wallenstein, 2007). Stress tolerance
mechanisms therefore become hugely significant in the ecological context, as allocating energy
and resources towards survival and maintenance simultaneously diverts them from growth,
and so comes at the momentary expense of competitiveness (Rillig et al., 2015). Populations that induce cross-tolerance or anticipatory mechanisms to respond to possible future stress may do so at the risk of other populations enjoying a growth advantage. Similarly, populations that experience a rapid loss of numbers due to unpreparedness in the face of disturbance are also vulnerable to becoming overwhelmed by their competitors. In fact, interspecific competition has been shown to become more significant as the level of environmental disturbance increases, controlling species extinction events as well as community richness (Violle, Pu and Jiang, 2010). Schimel et al. (2007) argue that environmental stress has the capacity to structure compositions and functions of soil communities to the point where we should redefine our current concept of process-based ecological functional groups (e.g. nitrifiers and denitrifiers) to include environmental stress responses, such as ‘drought tolerators’. The differing ability of microbes to respond to abiotic stress in the biotic context of competition is therefore a pertinent avenue of research.

Wesener and Tietjen (2019) developed a theoretical model to simulate microbial protective mechanisms in their respective environmental settings, and found that the community context can significantly alter the costs and benefits of induced stress responses. Cross-tolerance and anticipation provide a larger benefit for species in their natural niche compared to in a laboratory setting: an early response to future stress means more time to acquire nutrients and space compared to other populations, regardless of how quickly the competitors can activate their stress responses (Wesener and Tietjen, 2019). The costs and benefits of induced cross-protection are also inherently linked to the life strategy of the microorganism in question. Hilker et al. (2016) evoke the classic r- and K-selection concept to frame these considerations, where r-strategists exhibit rapid development and a high reproductive rate, while K-strategists employ the opposite approach, with a slow development and low reproductive rate. These adaptations can be viewed through the nature of the environmental disturbance: for environments characterised by frequent disturbances and a high degree of competitiveness, the early response is superior; here, r-strategists can be expected to perform well in competition, and induced cross-tolerance is often not stressor-specific. However, when observing systems prone to longer durations of more predictable stress events, a stronger stress response confers greater competitive advantages; here, K-strategists may enjoy success, inducing a strong and specific stress response (Wesener and Tietjen, 2019; Hilker et al., 2016). It should be noted that
the r- and K-selection concept translates to nature as a continuum, rather than a dichotomous either-or; different populations of the same species can even display r/K strategy plasticity (Hilker et al., 2016). Furthermore, the r- and K-selection theory was designed for animal life strategies: some researchers believe that the life strategies of environmental microbes shares more similarities with those of plants. Therefore, the three-dimensional Competitor-Stress tolerators-Ruderal (C-S-R) framework, designed for plants, may be more applicable to bacteria (Ho et al., 2012). Microbial competition is clearly distinguished by incredibly complicated interactive networks; elucidating these interactions, and, in particular, understanding how environmental disturbance and induced cross-tolerance impact microbial community dynamics, could prove to be pivotal for many different disciplines. Exploiting microbial communities in this way has numerous potential applications, such as wastewater treatment, bioenergy production (Werner et al., 2011) and biocontrol in agriculture (Cheng et al., 2016).

1.4.2 The nature of microbial competition

Before endeavouring to unravel complex interspecies mechanisms and interactions, it is perhaps beneficial to first consider the idea of intraspecies processes; bacteria within a single-species population also engage in competition. In heterogenous environments, bacteria will eventually accumulate mutations which, through selection pressures, give rise to variants that are better adapted to specific ecological niches (Hibbing et al., 2009). For example, Pseudomonas fluorescens undergoes adaptive radiation in static liquid cultures, where a portion of the population diversifies to overproduce extracellular polysaccharides (EPS), enabling them to float on the surface of the medium and occupy that oxygen-rich niche. However, if this sub-population becomes too dominant, the surface layer of EPS will become too thick and sink down to the bottom of the medium (Rainey and Travisano, 1998). In this case, intraspecies competition drives spatial differentiation. Diverse competitive strategies can also be unearthed through intraspecies interactions, where cooperation between individuals can confer a species with a competitive advantage: indeed, the competitive mechanisms available to a single cell in isolation may differ from those available to an individual within a larger single-species population (Hibbing et al., 2009). Quorum sensing is a form of bacterial communication whereby individuals can coordinate their gene expression; this is achieved through the secretion of diffusible signalling molecules which are sensed by other cells, and upon reaching a density-dependent threshold, the whole population initiates a concerted
action (Whitehead et al., 2001). Mavridou et al. (2018) describe a colicin-toxin mediated collective behaviour in *E. coli*, wherein cells are able to initiate massive coordinated attacks on competing strains; the authors liken this complex antagonistic response to alarm calling or pheromone secretion in social animals.

Interspecies interactions, while sometimes relying on syntrophic and otherwise mutually beneficial relationships, rarely achieve comparable levels of cooperation and coordination. This could be due to a conflict of evolutionary interest: cells that compete within their own species are not truly competing in an evolutionary sense, and so it makes sense to invest rather in cooperative phenotypes, such as the production of enzymes that release nutrients from the environment for all to enjoy (Cornforth and Foster, 2013). In a mixed-genotype population, there would be an incentive for different species to reap the benefits of this enzymatic nutrient preparation without contributing to the energy expenditure required to mobilise them; thus, ‘cheating’ prevents effective interspecies cooperation, and competition becomes predominant (Travisano and Velicer, 2004). Cheaters can emerge even in populations differing only through a few mutations: for instance, consider the *P. fluorescens* example mentioned above; in this system, cheaters eventually colonised the broth surface niche, enjoying the oxygenic benefits of growing in the surface biofilm without themselves contributing to the production of ESP (Rainey and Rainey, 2003). Once a bacterium has colonised a favourable niche, it is therefore usually in their best interest to prevent intrusion of potential competitors (Hibbing et al., 2009).

Microbial community assemblages are formed through many different processes. One of these is habitat filtering, wherein phylogenetically related species sharing similar traits are expected to co-occur in the same environments due to their inability to adapt to abiotic conditions of different habitats (Koeppel and Wu, 2013). Interspecific competition is subsequently locally stimulated due to the principle of competitive exclusion, originally postulated by Gause in the 1930s after a series of co-culture experiments in which two closely-related species of yeast were grown together, resulting in the subjugation of one of the species (Gause, 1932). Competitive exclusion dictates that the more closely related two species are, the greater their similarity in niche preference and therefore the more intense the competition between them (Faust and Raes, 2012). These two diametrically opposed processes can operate simultaneously, with the relative strength of each force in relation to the other influencing the overall community assembly. Coexistence of competitors can eventually develop through
spatial separation, such as when millions of competing cells expand outwards onto an agar surface; competitive exclusion eliminates much of the original population, but an equilibrium forms through development of separate clonal patches. The net effect of competition can therefore be said to be an overall reduction in diversity and increase in ecological stability (Ghoul and Mitri, 2016).

1.4.3 Competitive strategies
At the heart of all microbial competition lies the struggle for nutrients and space. Nutrients such as carbon, nitrogen, phosphorous, etc. are unevenly distributed along environmental gradients, meaning that spatial positioning of bacteria is not a trivial affair; colonisation of nutrient-rich zones could be the difference between survival and death, resulting in conflict as microbial groups expand in space and attempt to colonise areas in which nutrients are more abundant before other species can utilise them (Ghoul and Mitri, 2016). Competition for these two limiting resources can be broadly divided into two separate mechanisms, namely exploitative competition and interference competition. Exploitative competition is characterised by indirect interactions, whereby a species rapidly uses up limiting resources or colonises an abundant niche, thereby preventing another species from doing the same (Hibbing et al., 2009). A simple example of this lies in the inherent trade-off between the rate and yield of ATP production. The rate of oxidative respiration rapidly becomes saturated at high levels of resource or low levels of oxygen, meaning that if organisms wish to increase the rate of their nutrient uptake, they must also employ the fermentative pathway. This increases the rate of ATP production and nutrient uptake, while concomitantly lowering the total energy yield, as fermentation only yields 2 mol of ATP per mole of glucose compared to around 32 mol ATP obtained via oxidative respiration (Pfeiffer, Schuster and Bonhoeffer, 2001). So, by sacrificing efficiency of energy production, bacteria can attempt to quickly gather up available nutrients in an example of exploitative competition.

Interference competition, on the other hand, is associated with direct, antagonistic interactions between competitors, with the winner acquiring the limiting resource (Hibbing et al., 2009). The production and secretion of toxins and other bactericidal/bacteriostatic molecules in order to destroy or inhibit competitors is perhaps how we initially picture competition between microbes to play out. Indeed, there are countless examples of microbial weapons, which does suggest that they are instrumental to invasion and competition in
environmental contexts (Granato, Meiller-Legrand and Foster, 2019). For instance, the type VI secretion system (T6SS) in gram-negative bacteria shoots a toxin-carrying needle into the target cell in a way that is analogous to the mechanism of a contractile bacteriophage tail (Ho, Dong and Mekalanos, 2014). This has been implicated in interference competition, since species with the system could outcompete and overwhelm other species, while T6SS mutant strains were rapidly displaced (Schwarz et al., 2010; Anderson et al., 2017).

Another important microbial feature to note when discussing competitive strategies is motility: microbes often utilise their capacity for locomotion to compete against other species, or conversely to avoid competition (Hibbing et al., 2009). For instance, in a co-culture experiment conducted by An et al. (2006) investigating interactions between Pseudomonas aeruginosa and Agrobacterium tumefaciens, motility was found to be a critical factor in the competitive success of the former. Motile P. aeruginosa dominated competition by ‘blanketing’ A. tumefaciens biofilms, while mutants devoid of the locomotive machinery were impaired in their blanketing ability and subsequently defective in competition. The authors reported that A. tumefaciens also used motility, but instead as a means of evading co-culture biofilms. In a different study, co-culture of Pseudomonas sp. AD21 and Pedobacter sp. V48 under carbon-limitation resulted in the induction of gliding motility in the latter strain. Pedobacter sp. V48 was the inferior competitor of the two, and so this gliding motility behaviour was suggested to be a means of escaping competitive interactions and exploring for resources in other areas (Garbevo and de Boer, 2009). Bacteria have evolved many different strategies to move over surfaces; these include twitching motility, utilising type IV pili; gliding motility, which has multiple mechanisms; and swarming motility, where numerous flagella are used to spread over moist surfaces (McBride, 2001).

1.4.4 Biofilm formation

Motility is a powerful strategy commonly employed by microbes to explore and compete with others for nutrients and space: however, the dominant lifestyle of bacteria in environmental ecosystems (as well as human hosts) is considered to be as part of a biofilm, a non-motile polymeric surface-associated community (Ren et al., 2014). They are therefore central to microbiology, and yet relatively little is understood about biofilm formation in the natural context, as genetic and biochemical biofilm mechanisms are incredibly variable across different strains and environmental conditions (Oliveira et al., 2015). Despite the underlying processes
remaining elusive, biofilm formation is generally understood to be a fundamental survival strategy induced when bacteria confront environmental stimuli, promoting tolerance to a wide range of stressors, and providing better access to nutrients (Du et al., 2020; Lories et al., 2020). This is largely due to the matrix of extracellular polysaccharides (EPS) surrounding the bacterial aggregates, allowing the population to adhere to each other and to solid surfaces (Guttenplan and Kearns, 2013). The barrier properties of the EPS matrix can neutralise or ‘dilute’ biocidal agents to sublethal concentrations before they can reach individual cells; these same properties protect cells against UV light and dehydration (Hall-Stoodley, Costerton and Stoodley, 2004). EPS-based volume expansion can often confer a competitive advantage, i.e. through production of large amounts of extracellular polymer, cells in the biofilm can gain preferential nutrient access by rapidly colonising surfaces when nutrients diffuse from below (Schluter et al., 2014).

Since cells in biofilms are sessile, biofilm formation and motility are often considered to be opposing mechanisms; indeed, in many organisms, the genes required for biofilm formation and flagellar motility are inversely regulated (Hölscher et al., 2015). The choice between sessile and motile lifestyles is an important one for bacteria, often dependent upon the environments in which they thrive and situations they are in. As such, bacteria may select between motility and biofilm formation as distinct survival strategies (Verstraeten et al., 2008). In reality, however, this relationship is often not so antithetical; Hölscher et al. (2015) report that, while motility is not crucial for biofilm formation in B. subtilis and P. aeruginosa, it is essential for competitive fitness because motility-deficient mutants exhibited delayed biofilm formation and were subsequently overwhelmed in competition experiments. In other bacterial systems, motility is required for biofilm formation, but is later inhibited to form mature biofilms; this suggests that maintenance of motility may destabilise the multicellular aggregates, perhaps due to cells detaching from the surface community and swimming away (Guttenplan and Kearns, 2013; Du et al., 2020). Adhesion is critical to maintain the integrity of the biofilm, and has been found to be important for the outcome of competition in mixed-species biofilms; adhesive genotypes are more likely to dominate the community (Schluter et al., 2014).

Once a bacterial population has established itself in a favourable location, long-term persistence requires mechanisms for preventing other species from intruding. For instance, adhesins prevent displacement by invaders, and the EPS matrix can smother and starve
competitors (Ghoul and Mitri, 2016). Conversely, microbes wishing to colonise a new niche must employ various strategies to clear out current residents: \textit{P. aeruginosa} produces rhamnolipid to stimulate dispersal of other species from their respective biofilms (Hibbing \textit{et al.}, 2009). Thus, ecological competition within biofilms is often intense, and competition has been shown to actively promote biofilm formation (Lories \textit{et al.}, 2020). Co-culturing strains of \textit{P. aeruginosa} leads to increased biofilm, but this is not due to cooperative interactions, as one of the strains is largely eliminated in the mature biofilm (Oliveira \textit{et al.}, 2015). Induction of biofilm formation in the face of competition is likely due to increased tolerance to stressors like antibiotics and other toxins produced by competing strains; as an example, the T6SS mechanism mentioned earlier is inhibited by biofilm-dependent clonal patches (Lories \textit{et al.}, 2020). Interestingly, the mechanisms with which bacteria sense competition have been linked to their stress responses. Cornforth and Foster (2013) put forth the competition-sensing hypothesis, arguing that bacteria can use stress responses to sense harmful effects of other cells and therefore directly detect ecological competition. Instead of detecting chemical cues, which would be unique to different genotypes, stress response-mediated detection provides a more general system that directly responds to harmfulness. For instance, nutrient limitation induces a stress response; since this is inherently linked to competition, it would be logical to use it as a cue for the presence of a competitor (Cornforth and Foster, 2013). Research has shown that nutrient limitation drives a range of responses, including increased biofilm formation, virulence and antibiotic tolerance; these responses are all associated with ecological competition (Lories \textit{et al.}, 2020).
2 Objectives

The radiation and cold resistance exhibited by many bacteria of the genus Hymenobacter is well documented. Research into these traits could be of great biotechnological value, but characterisation of further stress tolerance is needed to expand our current understanding of the life strategy and behaviour of these microorganisms in their natural ecological context. The main aim of this project is therefore to assess the ability of the four Icelandic Hymenobacter strains in the University of Akureyri strain collection to tolerate osmotic stress, a stressor that they may commonly face in their natural environment. Investigations into the ecology of environmentally isolated bacteria would be incomplete without considering the larger community context. Bacteria live in complex associations with other diverse species, and their interactions shape the overall functional properties of their environment. Studying interspecific interactions can simultaneously shed light on the competitive strategies employed by bacterial community members, further increasing our understanding of the life strategy of the Hymenobacter isolates that are the object of the current study.

The overall goals of the project are therefore twofold: to determine the extent of the osmotic stress tolerance of the Hymenobacter strains, and potentially begin to elucidate the mechanisms they use to respond to this abiotic stressor; and to begin to assess the nature of their interspecific interactions with other strains isolated from the same environments, providing a fundamental groundwork for further studies on the competitive strategies and functional roles of these strains in their respective environments.

Based on these main objectives, the following research questions were proposed:

1. Are the Icelandic Hymenobacter strains in the UnAk strain collection able to tolerate osmotic stress down to aw 0.950?
2. Do the Hymenobacter strains fare well in competition with co-isolated strains?
3. Will co-culturing the Hymenobacter strains with co-isolated strains reveal any interspecific interactions?
3 Materials and Methods

3.1 Culture conditions and growth considerations

The four strains that were the object of this study (H16F320, JF1031, FN1603 and LW0504) had been stored at -80°C in Eppendorf tubes containing a 30% (w/v) glycerol solution. Prior to their use, they were allowed to thaw at room temperature for 5 minutes, and were then vortexed to resuspend the samples. Using an inoculation loop, the cell suspensions were transferred onto the surface of Reasoner’s 2 (R2A) agar plates in duplicate. Reasoner’s medium R2A is generally used for culturing bacteria from oligotrophic environments such as river water or barren soils (Zimbro et al., 2009), and previous studies have found this medium to be appropriate for the culturing of the strains in question.

After 8 days of culturing at room temperature, strain FN1603 showed no sign of growth, while strains H16F320 and LW0504 showed signs of contamination. Thus, FN1603 was replated from a reserve Eppendorf solution in the UnAk strain collection, and the other three strains were sub-cultured onto new plates in order to obtain pure, uncontaminated cultures. After 5 days, FN1603 still showed no signs of growth, and repeated attempts to revive this strain throughout the study resulted in only marginal success; thus, the following work was largely performed in the absence of strain FN1603. Throughout the duration of the study, strains H16F320, JF1031 and LW0504 were regularly sub-cultured to maintain the strains; they were at certain points also re-inoculated onto R2A plates from the original glycerol stocks when sub-culturing resulted in diminished growth.

All strains were grown at room temperature inside a dark cupboard. To confirm the optimum temperature for simultaneous growth of all the strains, agar plates divided into sections were inoculated with the strains and then incubated at 5°C, 10°C, 15°C, 22°C, 30°C, 35°C and 40°C for 7 days. The optimum temperature was then assessed via visual inspection of growth of each strain.
3.2 Osmotic stress tolerance test

3.2.1 Strains used

For the osmotic stress tolerance investigation, two experiments were performed in total. In each experiment, the Bioscreen could accommodate 5 strains. Since strain FN1603 did not grow adequately for the duration of the study, the three *Hymenobacter* strains tested were H16F320, JF1031 and LW0504. Each run of the experiment therefore had the capacity for two more strains; in the first run, strains MAS034 and MAS040 from the UnAk strain collection were used. Previous sequencing of their 16S genes placed them in the genus *Dyadobacter*, with both of the strains being phylogenetically most similar to the species *D. hamtensis*. These strains were isolated in 2014 from the terricolous lichen species *Peltigera membranacea* in the East of Iceland. The two strains MAS034 and MAS040 were revived from glycerol stocks by plating them on R2A plates at room temperature in duplicate, 6 days before the experiment was conducted. For the second run of the experiment, the two strains AR0120 and AR0315 were used as controls. These correspond to a halophilic *Salinicoccus* sp. and a halotolerant *Bacillus* sp. respectively. Previous sequencing of their 16S genes placed them as members of the species *S. roseus* and *B. indicus*. Both strains were originally isolated in 2009 from the lichen species *Lecanora helicopis* (AR0120) and *Hydropunctaria maura* (AR0315), both of which were sampled in Sílastaðatangi, Eyjafjörður, Iceland (Sigurbjörnsdóttir et al., 2014). The strains were taken from glycerol stocks and plated on Marine Agar (Zimbro et al., 2009) before culturing at room temperature, 6 days before the start of the experiment.

3.2.2 Preparation of stock solutions

In order to investigate the effect of lowering water activity on the growth of the *Hymenobacter* and control strains, the humectants NaCl, sucrose and glycerol (85%) were used to decrease the aw of liquid R2A broth. Based on preliminary experimental data and calculations, 8.5 g NaCl were dissolved in 91.5 mL R2A; 80 g sucrose in 100 mL R2A; and finally, 25 mL glycerol in 92 mL R2A. Preliminary water activity measurements indicated that these solutions would provide R2A broth solutions with aw values of 0.949, 0.950 and 0.949, respectively. After dissolving the solutes, using a hot plate stirrer if necessary, the three solutions were then autoclaved for 15 minutes at 121°C (standard media protocol). The sterile broth stock solutions were then left overnight at room temperature.
3.2.3 Growth experiment under varying water activity levels

From preliminary calculations, using the 0.950 aw stock solutions as the most concentrated media, sub-stock solutions with water activity values ranging from 0.950-0.990 could be prepared by diluting the stock solutions with R2A broth. In the first run of the experiment, varying volumes of each stock solution were diluted with pure R2A broth in 3 separate 50 mL beakers for a final volume of 25 mL in each case, in order to create solutions with a range of different water activities. Each solution was mixed thoroughly before use, and aseptic technique was used to minimise risk of contamination. A small amount (2-3 mL) of each of the 12 solutions (4 for each humectant) was extracted in order to measure the water activity. For the original stock solutions, 5 samples were taken to measure the water activity, while for the diluted solutions, the water activity measurements were performed in duplicate. For the NaCl media, the $a_w$ values were, on average, as follows: 0.946, 0.955, 0.967, 0.975 and 0.989. For the sucrose media, the $a_w$ values were: 0.939, 0.955, 0.967, 0.979 and 0.989, and for the glycerol media, the values were 0.943, 0.955, 0.963, 0.975, and 0.989. The highest water activity values in each case (0.989) refer to pure R2A broth, that is, without any added humectant.

For the second run of the experiment, the sub-stock solutions were prepared at the same time as the stock solutions, i.e. the day before the experiment was conducted, so that they could be autoclaved and the dilutions would therefore not have to be performed aseptically. Here, 50 mL of each solution were prepared in autoclavable Erlenmeyer flasks, and 2-3 mL were removed before autoclaving in order to measure the water activity, which was performed in duplicate for each solution. For the NaCl media, the $a_w$ values were, on average: 0.950, 0.960, 0.968, 0.987 and 1.003. For the sucrose media, the values were 0.951, 0.961, 0.967, 0.980, and 1.003, and for the glycerol media, they were 0.954, 0.962, 0.975, 0.978 and 1.003. The highest water activity values in each case (1.003), again, refer to pure R2A broth.

After preparing the solutions, a volume of 400 µL was pipetted into the relevant wells of two 100-well microtiter plates. For each strain, there were 12 media solutions containing different concentrations of NaCl, sucrose and glycerol, as well as one solution of pure R2A broth; each growth condition was prepared in triplicate, meaning 39 wells were occupied by each of the 5 strains, for a total of 195 wells. Each well was then inoculated with the appropriate bacterium, by transferring a small amount of stationary-phase *Hymenobacter* or control strains.
from R2A and Marine agar plates using a wire loop in the first run of the experiment, and sterile toothpicks in the second run. Cell suspensions calibrated to a specific OD value would be more ideal in terms of reproducibility, but since *Hymenobacter* strains produce a large amount of extracellular polymers they tend to clump together, meaning that in solution, they will not be evenly distributed. It was therefore decided that inoculating the wells from solid cultures was more appropriate, taking care to transfer only a very small amount into each well. After inoculating each well with the respective bacterium, the microtiter plates were placed in the Bioscreen, where they were cultured at 23°C for 7 and 8 days (experiment 1 and 2, respectively) with growth being monitored turbidometrically by scanning the plates at 600 nm every 15 minutes.

### 3.2.4 Data and statistical analysis

Three growth curves were obtained for each condition, which were averaged into one value. From this value, the optical density measured in the blank wells, i.e. wells containing only liquid media, was subtracted. Line graphs were plotted from each growth curve in Excel. Statistical analysis was performed using the student’s T-test function in Excel: for each bacterium, growth curves at different water activities were compared with the respective growth curve under standard conditions (i.e. no water-activity lowering humectant). For this analysis, the data for each replicate growth curve was normalised to overcome differences in the initial inoculum. Thus, data from the first two hours of the experiment was removed to account for stabilisation of the OD levels before adjusting each curve to start at the same level of absorbance. After this, T-tests were performed on every data point against every respective data point on the standard conditions curve. A confidence level of 95% was used, i.e. resulting p-values < 0.05 were deemed to be statistically significant.

The growth curves were also analysed in RStudio using Growthcurver, a package developed by Sprouffske and Wagner (2016) which fits experimental growth curves to the logistic equation in order to summarize growth characteristics such as intrinsic growth rate (*r*) and carrying capacity (*K*). Briefly, the Growthcurver package was installed before reading the data from the osmotic stress experiments into the program. To remove background absorbance signal due to the media, the “blank” method in Growthcurver was used. This subtracts the values in a blank well, containing only media, from all other wells at the same timepoints, ensuring that even if
media precipitates over the course of the growth curves, the background absorbance will be removed. The SummarizeGrowthByPlate function was then used to calculate growth parameters, and then the data table was saved to a tab-separated file which was then imported into Excel. The growth parameters from the triplicate growth curves for each condition were averaged into one, and T-tests were performed where applicable to determine whether the differences in these parameters were statistically significant (p < 0.05).

3.3 Preliminary determinations of competitive strategies/interspecific interactions

3.3.1 Strains used
In order to examine interspecific interactions between the *Hymenobacter* sp. under study and other strains in similar ecological contexts, all strains from the UnAk collection that had been isolated from the same samples as the *Hymenobacter* sp. were cultured. Thus, for LW0504, other strains from the sample LW05## were retrieved from freezer storage; this comprised strains 01, 02, 03, 05, 06, 07, 08 and 09. For JF1031, strains belonging to JF10## were 20, 21, 22, 23, 24, 25, 26, 28, 30, 33 and 34. For H16F320, strains belonging to the sample H16F3## were 01, 02, 14, 15, 17, 19, 20, 21, and 23. Finally, for FN1603, strains also belonging to FN16## included 05, 06, 07, 08 and 09. All strains had been stored at -80°C in Eppendorf tubes containing a 30% (w/v) glycerol solution. After thawing at room temperature, the tubes were vortexed to resuspend the samples, and the cell suspensions were then transferred onto the surface of R2A agar plates in duplicate, using an inoculation loop. The strains were incubated at room temperature. These optimum culture conditions for the strains were unknown; to simplify things for the co-culture experiment, only strains that grew at room temperature and on R2A media were used for further study. Thus, the strains that grew under these conditions were: LW0501, LW0502, LW0503, LW0505, LW0506, LW0507 and LW0508; JF1020, JF1021, JF1022, JF1023, JF1025, JF1030, JF1033 and JF1034; H16F314 and H16F315; FN1605, FN1606 and FN1608.

3.3.2 Media preparation
In order to investigate interspecific interactions, several varieties of solid R2A agar were prepared. 2L of regular R2A were made by dissolving 18.2g dehydrated R2A media/litre distilled water. 1L 0.5% agar was prepared by following the recipe for R2A (Reasoner and
Geldreich, 1985), but instead of 15g agar, corresponding to 1.5% concentration, only 5g were used. This media was used for the co-culture experiment to determine whether the presence of other strains would induce motility in the *Hymenobacter* sp. Similarly, 1L of a slightly nutrient-deficient variant of R2A was also prepared by following the recipe for R2A, but omitting the starch. This media was used in order to determine whether nutrient limitation would induce more intense competition between the strains under study. All media was prepared by adding the relevant ingredients to the appropriate volume of distilled water, before boiling the water briefly in order to ensure that the contents had dissolved. After this, the media was autoclaved for 15 minutes at 121°C, before being cooled down to 50°C in a water bath at which point the media could be poured into petri dishes. The agar plates were stored at room temperature in sealed plastic bags to prevent airborne contamination.

3.3.3 Co-culture interaction assessment
To assess for interspecific interactions, cross-spotting on agar plates was performed. This semi-quantitative assay is used to assess antagonistic and synergistic effects of two bacterial strains on one another. Each petri dish was marked with six evenly spaced dots (one dot every 1.4 cm) in a straight line across the centre of the plate. The plates were then rotated 90°C and this process was repeated, so that each plate contained 12 dots forming a cross. Along one of the straight lines, either strain LW0504, JF1031, H16F320 or FN1603 were stab-inoculated into each of the 6 dots using a sterile toothpick. Along the other line, one of the strains previously cultured for the co-culture experiment was inoculated in the same way. Each of the *Hymenobacter* strains were therefore inoculated along with every one of the strains isolated from the same sample, i.e. LW0504 was inoculated with LW0501, LW0502, LW0503, LW0505, LW0506, LW0507 and LW0508. Each of these combinations was plated in duplicate, on R2A plates. The plates were cultured for 8 days at room temperature.

This process was repeated for LW0504 and H16F320 and their respective co-isolated strains, and this time they were cultured on R2A, 0.5% agar R2A and starch-free R2A. Therefore, each strain combination was plated 6 times in 3 different types of media. The plates were incubated at room temperature for 8 days, before growth on each plate was examined.
3.3.4 Biofilm formation assessment

In order to determine whether the strains under study can form biofilms on polystyrene, 100 µL of the slightly nutrient-limited broth medium 0.5xTSB were pipetted into each well of a 100-well microtiter plate under aseptic conditions. Using sterile toothpicks, strains LW0504, JF1031 and H16F320 were inoculated into wells of the Bioscreen plate, as well as the co-culture strains that had grown at room temperature. Each strain was inoculated into 5 wells, and 20 strains in total were inoculated (FN1603 and the respective co-culture strains were not included due to poor growth) for a total of 100 wells. The strains were incubated at room temperature for 5 days.

After incubation, the liquid media was discarded from the wells and the plate was rinsed twice with distilled water to remove planktonic or loosely-bound bacterial cells. Following this, 125 µL of 0.1% crystal violet solution was pipetted into each well. After 15 minutes, the solution was discarded from the wells and the plate rinsed 4 times with distilled water. The plate was then left to dry overnight. Next, 125 µL of 30% acetic acid was pipetted into each well of the plate. After 15 minutes, the optical density was measured at 540 nm in the Bioscreen scanner. The average OD value of blank wells measured on a separate microtiter plate containing only 30% acetic acid were subtracted from all resulting absorbance values. These values were averaged into one and statistical analysis was done by performing T-tests using a 95% confidence level.
4 Results

4.1 Osmotic stress tolerance investigation

4.1.1 Preliminary optimal temperature determination

To determine the optimum incubation temperature that would allow for simultaneous growth of the microorganisms under study in the Bioscreen, a preliminary temperature test was conducted with the three *Hymenobacter* strains and *Dyadobacter* strains MAS034 and MAS040: the results are shown in Figure 1.

![Figure 1: Growth of strains after 8 days exposed to different temperatures. Strain 1 is H16F320; strain 2 is JF1031; strain 3 is LW0504; strain 4 is MAS034; strain 5 is MAS040.](image)

Clearly, the *Hymenobacter* sp. can not grow at temperatures above 30°C. Interestingly, strain H16F320 showed growth in all three replicates at 5°C but did not grow at 10°C. Neither strain JF1031 or LW0504 showed growth at 5°C or 10°C, but this is likely due to the relatively short (in terms of psychrophilic bacteria) incubation time of 8 days. At 15°C, all strains showed
relatively good levels of growth; strain H16F320 grew in all three replicates, while strains LW0504 and JF1031 only grew in 2 and 1 plates, respectively. At 23°C, one of the replicate plates exhibited growth of all strains simultaneously; overall, this temperature allowed for slightly better growth than 15°C, and so this temperature was chosen for the following experiments.

4.1.2 Osmotic stress tolerance experiment 1
Each bacterium under study was subjected to a range of water activities from 0.989-0.939. The water activity was lowered either with NaCl, sucrose or glycerol. For each of these conditions, a separate growth plot was produced, showing how each bacterium responds to lowered water activity and whether the humectant induces any specific solute effects.

![Growth curves from the first run of the osmotic stress experiment for Hymenobacter strains H16F320, JF1031 and LW0504.](image)

Figure 2: Growth curves from the first run of the osmotic stress experiment for Hymenobacter strains H16F320, JF1031 and LW0504. On the plots, the y-axes represent the average optical density, measured at 600nm, while the x-axes represent time, measured in hours. The first row shows H16F320: 2A has NaCl as the water activity-lowering humectant, 2B has sucrose, and 2C has glycerol. The second row shows the results for JF1031, where plot 2D contains NaCl as the humectant, 2E has sucrose, and 2F has glycerol. The third row shows the results for LW0504, where plot 2G contains NaCl as the humectant, 2H has sucrose, and 2F has glycerol. The colours of each of the curves represents growth at a particular water activity level, indicated in the legends for each column.
Inspecting the growth curves for each strain under standard conditions (0.989 aw, shown in every plot for comparison), it appears that strain JF1031 has a lag time about twice the length of the other two strains. Only after about the 50 hour mark does strain JF1031 begin to grow under standard conditions, while strains H16F320 and LW0504 have a lag time of around 25 hours. Using the open-source R package Growthcurver, these experimentally determined growth curves were fitted to a basic form of the logistic equation, which determines the number of cells \( N_t \) at time \( t \). Thus, for each curve, various growth parameters were determined using the implementation of the non-linear least-squares Levenberg-Marquardt algorithm (Sprouffske and Wagner, 2016). The carrying capacity (\( K \)) is the maximum possible population size in a particular environment, represented by the sigmoidal plateauing of the growth curves as the stationary phase is reached; these values were determined, according to the best fit of the logistic equation, to be 0.344, 0.381 and 0.288, on average, for H16F320, JF1031 and LW0504, respectively. These values represent optical density, rather than absolute bacterial counts. The intrinsic growth rate, \( r \), is another metric calculated by the Growthcurver R package; this represents the growth rate that would occur if there were no restrictions imposed on total population size (Sprouffske and Wagner, 2016). Under standard conditions, H16F320 has an intrinsic growth rate of 0.081; JF1031 has \( r = 0.069 \) and LW0504 has \( r = 0.044 \). T-tests performed did not indicate that the differences in these values were statistically significant. Another important parameter calculated using the R Growthcurver package is the area under the growth curve (AUC); this metric integrates information from the logistic parameters (\( K, r \) and \( N_0 \)) into a single value, emphasising growth rate and carrying capacity (Sprouffske and Wagner, 2016). The AUC for H16F320, JF1031 and LW0504 at \( a_w \) 0.989 was 42.9, 31.2 and 30.3, respectively. Interestingly, t-tests indicated that the difference between the AUC for H16F320 and the other two strains was statistically significant (\( p = 0.01 \) for JF1031 and \( p = 0.02 \) for LW0504). The difference between JF1031 and LW0504 was not significant, as the AUC values are very similar. It is worth noting that while the AUC metric suggests that H16F320 exhibits the ‘best’ growth under standard conditions, manual inspection of the growth curves reveals that strain JF1031 has not yet reached the stationary phase and so the area under the curve does not give the whole picture. The carrying capacity \( K \) of JF1031 is greater than that of H16F320, showing how even though it is a slower-growing strain, it will eventually reach greater
population counts under these conditions. Strain LW0504, however, has both the lowest growth rate and carrying capacity under these conditions.

The curves obtained under standard conditions reveal interesting characteristics about the nature of growth for these strains; however, under all other conditions, the strains did not exhibit any measurable growth. The one exception to this is LW0504 in Fig. 2l, where glycerol was used to adjust the $a_w$ to 0.955. However, since no growth occurred at $a_w$ 0.963 or 0.975 when glycerol was also used, this growth can be attributed to contamination; this is further supported by the fact that growth only occurred in one of the three triplicates. Care must be taken when interpreting the metrics obtained using Growthcurver for these curves, as they do not fit the logistic model of growth. As such, the growth rate $r$ of H16F320 in Fig. 2A is 0.006 for $a_w$ 0.975, but 0.431 for $a_w$ 0.967. This value, while clearly erroneous, does not skewer the results when combined with the carrying capacity $K$, which under these two conditions are 0.007 and 0.004, respectively. These two parameters are both required to give a clearer picture; combined into one value in the form of AUC, $a_w$ 0.975 has an area of 2.6 while $a_w$ 0.967 has an area of 0.7; compared with the AUC under standard conditions (42.9), these conditions are clearly not conducive for growth. The $r$, $K$ and AUC values under the other conditions are displayed in supplementary tables in Appendix 2. T-tests performed indicate that the differences in AUC values between growth under standard conditions and all other conditions are statistically significant in every case ($p < 0.05$).

Analysis of statistical significance was accomplished by performing Student’s t-tests for every single timepoint on every growth curve from Fig. 2 against the standard condition curve for the respective strain. These results are presented in Figure 14 in Appendix 1. Statistical analysis of the growth curves from Figures 3, 4 and 5 are also presented in Appendix 1 Fig. 15, 16 and 17.
Growth curves from experiment 1 were also generated for *Dyadobacter* strains MAS034 and MAS040, shown in Figure 3. The standard condition curves for these strains seem to experience two log phases, suggestive of diauxic growth. The intrinsic growth rate $r$ modelled in RStudio was determined to be 0.043 and 0.006 for MAS034 and MAS040 under standard conditions, respectively. The carrying capacity of these two curves was determined to be 0.205 and 0.102. Comparing the growth rate, MAS034 seems to grow around 7 times faster than its counterpart, while it reaches a maximum population around twice the size of MAS040. It is possible that the diauxic growth pattern of both strains could present problems when fitting the curves to the logistic equation in Growthcurver, and so these metrics shouldn’t be scrutinised too carefully. The AUC for the two strains is 46.47 and 14.31 under standard conditions, which suggests that strain MAS034 has a threefold higher capacity for growth than MAS040 under these conditions. It may be the case that for MAS040, standard conditions are not the optimum conditions for growth; Fig. 3F shows how MAS040 displays more effective growth at $a_w$ 0.975, with glycerol as the humectant. Here, the AUC is 37.59, which is higher than JF1031, LW0504 and MAS034 under standard conditions. MAS040 does not display these effects at similar $a_w$ levels with the other two humectants, however. T-tests between the AUC values of the Fig. 3F $a_w$ 0.975 growth curve and the standard conditions growth curve of the other strains (including MAS040) did not result in statistical significance ($p > 0.05$). This is due...
to the extreme distribution of the AUC values of the three MAS040 glycerol \( a_w \) 0.975 replicates (14.76, 13.0 and 84.96). It is perhaps most likely that the last replicate is due to contamination, suggesting that while MAS040 may be slightly tolerant of the presence of glycerol in the growth medium, it does not prefer it. This wide distribution is also exhibited in the standard conditions curve of MAS034; here, the AUC is on average 46.47 and the standard deviation is 36.25. As such, it can not be expected that any comparisons with this curve will result in statistical significance.

### 4.1.3 Osmotic stress tolerance experiment 2

![Graphs showing growth curves for different strains and humectants](image)

**Figure 4:** Growth curves from the second run of the osmotic stress experiment for Hymenobacter strains H16F320, JF1031 and LW0504. On the plots, the y-axes represent the average optical density, measured at 600nm, while the x-axes represent time, measured in hours. The first row shows H16F320: 4A has NaCl as the water activity-lowering humectant, 4B has sucrose, and 4C has glycerol. The second row shows the results for JF1031, where plot 4D contains NaCl as the humectant, 4E has sucrose, and 4F has glycerol. The third row shows the results for LW0504, where plot 4G contains NaCl as the humectant, 4H has sucrose, and 4I has glycerol. The colours of each of the curves represents growth at a particular water activity level, indicated in the legends for each column.

The second run of the osmotic test experiment largely constitutes a repetition of the first run, with the aim of producing reproducible results. However, several key differences exist between the two experiments; the largest difference is the identity of the two control strains, presented further below. Small differences in water activity levels of media solutions can also be identified.
between the two runs, and finally, this experiment was conducted for 187 hours compared to 169 hours in the first run. It is important to note the dip in optical density around the 150 hour-mark seen in every growth curve in Figure 4: this is due to the Bioscreen being turned off at this point. After immediately plotting the growth curves, it was decided to run the experiment for an additional 2 days since many of the curves had not reached the stationary phase. It is also important to note that the $a_w$ of R2A media containing no added humectant was in this case measured as 1.003; since pure water has an $a_w$ of 1.000, this is a strange result.

The intrinsic growth rates of strains H16F320, JF1031 and LW0504 were determined in R to be, on average, 0.065, 0.026 and 0.062 under standard conditions, respectively. Inspecting the standard growth curves (at $a_w$ 1.003) in Fig. 4A and 4G, LW0504 clearly has a higher intrinsic growth rate; perhaps the Growthcurver algorithm was disrupted by the OD dip at the 150 hour-mark. Looking at the standard deviations for these growth rates, H16F320 has a standard deviation of 0.012, compared to 0.002 and 0.003 for JF1031 and LW0504, respectively. In all likelihood, the actual growth rate of H16F320 is within the lower bounds of the estimate. The carrying capacity of the three strains was determined to be 0.193, 0.468 and 0.554, respectively. This is more in line with the growth curves; both H16F320 and LW0504 experience peaks in OD at around the points delineated by the $K$ values. Strain JF1031 exhibited comparatively slower growth, and had not yet reached the stationary phase after 187 hours. Thus, the maximum population size calculated by Growthcurver in Rstudio is useful here to simulate how large the population would eventually get; in this case it was determined to be, on average, 0.468. The AUC values are useful here to describe the growth of H16F320 and LW0504 in a single value; 17.28 and 43.24, respectively. However, it cannot be used to describe the growth profile of JF1031 because the curve does not reach the stationary phase; as such, the AUC of JF1031 is only 5.05. Of course, it can be used to compare growth of the three strains within the timeframe of the experiment. T-tests indicated that the AUC of JF1031 and LW0504 are significantly different ($p = 0.036$) while the other two combinations did not result in $p < 0.05$ (0.057 and 0.124).

The AUC of the standard conditions curves, however, are in almost all cases significantly different from the AUC of curves with lowered water activity. The exceptions to this are Fig. 4A $a_w$ 0.987 and 0.960 ($p = 0.054$; 0.059), Fig. 4E $a_w$ 0.951 ($p = 0.808$), Fig. 4G $a_w$ 0.968 ($p = 0.133$) and Fig. 4H $a_w$ 0.961 ($p = 0.197$). Interestingly, the Fig. 4A $a_w$ 0.968 curve shows slight growth.
after the 150-hour mark, with the $K$ value suggesting H16F320 will reach an OD of 0.036 at this water activity. The carrying capacity $K$ of H16F320 when sucrose is used to lower the $a_w$ to 0.980 is 0.055; for glycerol $a_w$ 0.975, $K$ for H16F320 is 0.041. These values, compared to 0.002-0.0012 at lower water activities, suggest that H16F320 may be able to tolerate very slight osmotic stress, even though growth was not exhibited in the latter two cases. In Fig. 4H, LW0504 seems to grow slightly at $a_w$ 0.961 with sucrose; this curve was among the few that were not significantly different from the standard curve, due to variations in the replicates; this is most likely caused by contamination. Also clearly contaminated are all three growth curves at $a_w$ 0.978 glycerol, which are all nearly identical; here, the stock solution containing the media itself was likely contaminated, rather than for instance the toothpick used to inoculate the bacteria. The curve at $a_w$ 0.975 in Fig. 4I is also most likely due to contamination, since only one of the three replicates exhibited growth. The only growth curve which could indicate tolerance to osmotic stress is in Fig. 4F, at $a_w$ 0.975. Here, glycerol is used to lower the water activity and JF1031 is the strain in question. Inspecting the curve, the rate of growth is identical to the standard conditions curve ($r = 0.026$), only with a longer lag time. The carrying capacity for this curve is 0.230, around half of that calculated for standard conditions (0.468).

![Growth curves for strains AR0120 and AR0315. On the plots, the y-axes represent the average optical density, measured at 600nm, while the x-axes represent time, measured in hours. The first row shows AR0120: 5A has NaCl as the water activity-lowering humectant, 5B has sucrose, and 5C has glycerol. The second row shows the results for AR0315, where plot 5D contains NaCl as the humectant, 5E has sucrose, and 5F has glycerol. The colours of each of the curves represents growth at a particular water activity level, indicated in the legends for each column.](image-url)
The two positive control strains AR0120 and AR0315 were chosen due to their halophilic and halotolerant natures. Looking at Figure 5, their standard conditions growth curves can clearly not be interpreted as comparable to the other strains, as here, standard conditions are not equivalent to optimum conditions. So, while strain AR0120 exhibits a very rapid growth rate of 0.173 under standard conditions, the carrying capacity $K$ is only 0.044, which is more than tenfold lower than the maximum population size of the other strains under standard conditions. Being a halophile, AR0120 experiences the largest carrying capacity at $a_w$ 0.987 adjusted with NaCl (Fig. 5A), $K = 0.617$. The carrying capacity goes down to 0.260 at $a_w$ 0.968, but then increases to 0.485 and then 0.591 at $a_w$ 0.960 and 0.950, respectively. It is therefore possible that at even lower $a_w$ levels, or rather even higher concentrations of NaCl, this strain could experience an even larger maximum population. When the other humectants are used to lower the water activity, strain AR0120 still exhibits growth, but to a much lower extent, with each water activity level producing a unique growth curve. This is what would be expected to occur in the *Hymenobacter* strains if they were tolerant to osmotic stress. The curves in Fig. 5C and 5F corresponding to $a_w$ 0.978, are due to contamination. Compared to AR0120, strain AR0315 is halotolerant and experiences more effective growth when sucrose and glycerol are present in the medium, rather than NaCl. Here, the maximum $K$ is at $a_w$ 0.980 with NaCl (0.412) and $a_w$ 0.975 with glycerol (0.325). At lower water activity levels, the carrying capacity decreases steadily. Interestingly, the AUC for both AR0120 and AR0315 at $a_w$ levels around 0.980 and 0.950 are very similar, both for NaCl and glycerol (see Table 7, Appendix 2). This was not observed for sucrose, where the maximum AUC was exhibited at $a_w$ 0.967 for AR0120 and 0.980 for AR0315. This may suggest that while both strains can grow unhindered at $a_w$ 0.980, they both could have a preference for $a_w$ around 0.950 or lower.
4.1.4 Comparison between the two growth experiments

Comparing growth curves of H16F320, JF1031 and LW0504 under standard conditions between the two experiments, it is interesting to note that for both JF1031 and LW0504, the growth curves generated become significantly different after about 100 hours. This is not the case for H16F320, for which the growth curves are more similar between the two runs of the experiment. It must be noted that while ‘standard conditions’ ostensibly are the same for the two experiments, since R2A broth without any added humectant was used in both cases, the water activity measurements do not indicate this. The table below summarises the growth metrics for these curves, calculated in RStudio.

Table 1: Parameters for growth under standard conditions (23°C, no humectant) for H16F320, JF1031 and LW0504, calculated in RStudio for experiments 1 and 2. In the ‘metric’ column, r represents intrinsic growth rate, K represents carrying capacity, AUC represents the total area under the growth curve, and Tgen represents the fastest doubling/generation time possible for the population, in hours. Columns labelled p contain results of t-tests conducted for each metric, indicating whether differences between the two experiments are significant. Statistical significance is indicated with a green colour (p < 0.05).

<table>
<thead>
<tr>
<th>Metric</th>
<th>H16F320</th>
<th>JF1031</th>
<th>LW0504</th>
</tr>
</thead>
<tbody>
<tr>
<td>r</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>r</td>
<td>1.12</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>K</td>
<td>0.36</td>
<td>0.32</td>
<td>0.36</td>
</tr>
<tr>
<td>AUC</td>
<td>47.86</td>
<td>37.25</td>
<td>43.46</td>
</tr>
</tbody>
</table>

Table 1 shows how the growth rates for H16F320 are not statistically different between the two experiments; neither is the doubling time, which is inherently linked to the growth rate. However, the difference between the carrying capacity is significant, which can be seen in Fig.

Figure 6: Growth curves under standard conditions (no added humectant) compared between experiments 1 and 2. The blue line represents growth at aw 0.989 during experiment 1, while the orange line represents growth at aw 1.003 during experiment 2. Fig. 6A shows H16F320, 6B shows JF1031, and 6C shows LW0504. The bars above the figures show statistical significance at each time point over the course of the experiments, where the colour green represents a significant difference between the two curves (p < 0.05).
where the maximum population size is clearly greater in experiment 1. The AUC is also significantly different, going from 42.86 in experiment 1 to 17.28 in experiment 2. Interestingly, the AUC is also significantly different for strain JF1031 between the two experiments, going from 31.16 to just 5.05, and yet neither the growth rate or carrying capacity are significantly different in this case. There is a large variability in the $K$ values for the three replicates of JF1031; while one of the replicates experiences a peak in maximum population size at around the same OD where the experiment ends, another replicate has the capacity to eventually grow to 5-6 times the population size shown in Fig. 6B. More replicates are required in order to obtain a clearer picture of the growth rate and carrying capacity for this strain. The differences in generation time for JF1031 are significant; the population in experiment 1 will double in 10.69 hours under no growth restrictions, while in experiment 2 the doubling time increases to 26.98 hours. For LW0504, differences in both $r$ and $K$ are significant. Although the intrinsic growth rates are similar (0.04 compared to 0.06), the replicates are consistent enough to warrant this small difference to be significant. The carrying capacity is more variable between the two experiments, with LW0504 exhibiting almost twice the maximum population size in the second run.
Comparing the standard conditions curves for each strain can reveal interesting characteristics of their growth, but does not involve their tolerance to osmotic stress. To demonstrate whether osmotic stress tolerance remains consistent through both runs of the experiment, the average area under each curve was plotted against water activity, shown in Figure 7. The average AUC was chosen since it summarizes both the intrinsic growth rate, \( r \), and carrying capacity, \( K \), into one variable, as well as taking into account factors such as initial population size, \( N_0 \). It must be noted that absolute values of AUC should not be compared between experiments, since experiment 2 was longer and so the area under the curves will also be larger. However, trends in AUC that occur with lowering water activity can be compared. For the three *Hymenobacter* strains in experiment 1, the AUC values peak at \( a_w 0.990 \) (standard
conditions) before sharply declining to 0 at $a_w$ 0.980-0.985, no matter which humectant is used. In experiment 2, the AUC values peak under standard conditions again ($a_w$ 1.003 in this case), but the subsequent decline occurs at different rates depending on the humectant. The decline with NaCl is sharpest, reaching 0 before $a_w$ 0.985 is reached. When sucrose is used, the AUC reaches 0 around $a_w$ 0.980 for all three strains, and when glycerol is used, H16F320 and JF1031 reach 0 around $a_w$ 0.970, and LW0504 around $a_w$ 0.965. In experiment 2, strains LW0504 and JF1031 sometimes show a small increase in AUC at lowered water activity levels, but this was attributed to contamination. Strains MAS034 and MAS040 are clearly not osmotolerant either.

The two control strains AR0120 and AR0315, corresponding to a halophilic *Salinicoccus roseus* strain and a halotolerant *Bacillus indicus* strain, exhibit specific solute effects. For AR0120, the maximum AUC is reached at $a_w$ 0.987 when NaCl is used, before decreasing rapidly and then increasing again, suggesting there is another peak below $a_w$ 0.950. When sucrose and glycerol are used, this strain does not fare as well, showing a limited AUC until $a_w$ 0.965 where growth begins to decline. The AUC begins to increase again as $a_w$ 0.950 approaches, suggesting this could be a desirable water activity for strain AR0120 and that the strain may have an optimum water activity below $a_w$ 0.950. In contrast, strain AR0315 shows the largest AUC when sucrose is used, at around $a_w$ 0.977. When NaCl and glycerol are used, peaks occur at $a_w$ 0.987 and 0.973 respectively, but the highest AUC values in both cases occur at the lowest water activity tested. Water activities around $a_w$ 0.975-0.80 appear to be conducive to growth of this strain, although it only appears to be the optimum when sucrose is used as the humectant, showing how specific solute effects are in play. In contrast, growth potential of AR0315 appears to increase below $a_w$ 0.950 when NaCl and glycerol are used as humectants, indicating that these solutes are not as inhibiting as sucrose for growth of this strain.
4.2 Preliminary investigations into competitive strategies/interspecific interactions

4.2.1 Preliminary co-culture test

In order to investigate the effects of culturing the *Hymenobacter* strains in the presence of other strains from the same ecological contexts, interaction assays were performed. Antagonistic or synergistic effects that the strains may have on one another should become more apparent the closer the cultures get to one another; the cultures on the edges of the plates can grow uninfluenced by the other strain, while those in the centre are forced to occupy the same space if they wish to grow outwards.

Figure 8: Co-culture interaction assay with JF1031 on R2A. The vertical inoculation lines contain JF1031, while the horizontal lines each contain a different strain isolated from the same sample as JF1031. In order, these strains are: JF1020, JF1021, JF1022, JF1023, JF1024, JF1025, JF1030 and JF1034.

A preliminary investigation was conducted to test the suitability of the *Hymenobacter* strains for such an assay. The relatively long lag times of the strains may have resulted in the other strains overwhelming the agar plates before the hymenobacters could even commence their growth. Figure 8 shows that this was not the case for JF1031, which had the longest lag time during the growth experiment above. However, most strain combinations did not result in any visible phenotypic alterations or growth reduction for JF1031; almost all strains confined themselves to the points where they were inoculated and did not attempt to expand outwards.
The one exception to this can be seen in Fig. 8D, where strain JF1023 was co-cultured with JF1031. Here there is a clear reduction in the growth of JF1031 as it gets closer to the other strain; it is also noteworthy to observe the aggression of expansion of strain JF1023 compared to the others.

Of the strains isolated from the same samples as H16F320 and FN1603, only two of each would grow under the same conditions as the *Hymenobacter* strains. The co-cultures presented in Figure 9 are not much different from the ones with JF1031, in that there don’t appear to be overly significant interactions between the strains. In Fig. 9 panel A, there is a slight growth reduction in H16F320 as it gets closer to H16F314, suggesting antagonism; the same can be said for panel B, but to an even lesser extent. Strain FN1603 did not grow very well along the whole line, but the growth was consistent at all points suggesting that it is not inhibited by the presence of the other strains. Strain LW0504 did not grow in any of the co-cultures.

4.2.2 Interspecific interactions of LW0504 and H16F320

To investigate the effects of slight nutrient deprivation and a lower agar concentration on competition, strains LW0504 and H16F320 were co-cultured with their co-isolated strains using various media compositions. It was found that in all cases, growth of LW0504 and H16F320 was reduced in the presence of their competitors. The extent to which the growth reduction of H16F320 was affected by media composition is summarised in Table 2.
Table 2: The effects of media composition and co-culture strain on the growth of H16F320. Growth reduction is represented as the scale factor of reduction from the culture at the edge of the plate to the culture in the middle of the plate. Growth under the agar is represented with ++ = strong growth, + = weak growth, - = no growth.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Media</th>
<th>Growth reduction</th>
<th>Growth under agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>H16F314</td>
<td>R2A</td>
<td>4.2 ± 2.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>starch-free R2A</td>
<td>7.5 ± 2.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.5% agar R2A</td>
<td>7.3 ± 3.0</td>
<td>-</td>
</tr>
<tr>
<td>H16F315</td>
<td>R2A</td>
<td>4.7 ± 2.8</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>starch-free R2A</td>
<td>5.3 ± 1.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.5% agar R2A</td>
<td>4.6 ± 2.4</td>
<td>-</td>
</tr>
</tbody>
</table>

When R2A was used as the medium, the presence of H16F314 caused, on average, a 4.2-fold reduction in growth for H16F320. This was calculated by measuring the relative sizes of the cultures at the edge of the plate, uninfluenced by the presence of a competitor, and the sizes of the cultures in direct competition in the middle of the plate: the ratio of the corresponding values was then calculated. Thus, when H16F320 was cultured on R2A with H16F314 as a competitor, the cultures at the end of the plate were 4.2 times larger than the cultures in the middle, on average. When starch-free and 0.5% agar R2A were used, growth was reduced even more, by a scale factor of over 7. This suggests that both nutrient limitation and increased mobility could have deleterious effects on competition for H16F320. These effects were not observed when H16F315 was used as the competitor strain, however; while growth of H16F320 was diminished in every case, it was reduced to a consistent extent, by a factor of 4.6-5.3.
Figure 10: Co-culture assay with H16F320 and H16F314. Panel A shows the two strains cultured on R2A; panel B on starch-free R2A; and panel C on R2A with 0.5% agar. Panel D shows close-ups of the twitching motility observed in panel A.

An incredibly interesting result of culturing H16F320 and H16F315 together was the apparent induction of twitching motility in the interstitial space between the agar and the bottom of the agar plate. Figure 10A and D show this growth, observed for both strains. Interestingly, the *Hymenobacter* cultures at the edge of the plate do not exhibit this growth to the same extent, suggesting that the presence of strain H16F314 could be required. This effect was not observed in starch-free R2A, or, interestingly, in 0.5% agar R2A. Fig. 10C shows growth on R2A with 0.5% agar; the colony morphology here is noticeably different, with a dense cellular mass in the centre of the colony and a smoother, lighter circle of growth diffusing outwards from the central body; no evidence of twitching motility was observed, however. In starch-free R2A, H16F314 colony morphology increases in size and changes to have more lobate margins, but twitching motility is lost for both strains.
Table 3: The effects of media composition and co-culture strain on the growth of LW0504. Growth reduction is represented as the scale factor of reduction from the culture at the edge of the plate to the culture in the middle of the plate. Growth under the agar is represented with ++ = strong growth, + = weak growth, + - = sporadic growth, - = no growth.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Media</th>
<th>Growth reduction</th>
<th>Growth under agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>LW0501</td>
<td>R2A</td>
<td>3.8 ± 0.3</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>starch-free R2A</td>
<td>6.2 ± 0.4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.5% agar R2A</td>
<td>18.5 ± 10.6</td>
<td>-</td>
</tr>
<tr>
<td>LW0502</td>
<td>R2A</td>
<td>6.6 ± 0.5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>starch-free R2A</td>
<td>3.3 ± 0.5</td>
<td>+ -</td>
</tr>
<tr>
<td></td>
<td>0.5% agar R2A</td>
<td>2.1 ± 0.8</td>
<td>-</td>
</tr>
<tr>
<td>LW0503</td>
<td>R2A</td>
<td>2.6 ± 0.5</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>starch-free R2A</td>
<td>1.8 ± 0.3</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.5% agar R2A</td>
<td>1.8 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>LW0505</td>
<td>R2A</td>
<td>6.8 ± 1.2</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>starch-free R2A</td>
<td>2.6 ± 0.4</td>
<td>+ -</td>
</tr>
<tr>
<td></td>
<td>0.5% agar R2A</td>
<td>5.6 ± 2.2</td>
<td>-</td>
</tr>
<tr>
<td>LW0506</td>
<td>R2A</td>
<td>4.7 ± 1.9</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>starch-free R2A</td>
<td>5.5 ± 0.4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.5% agar R2A</td>
<td>9.5 ± 1.1</td>
<td>-</td>
</tr>
<tr>
<td>LW0507</td>
<td>R2A</td>
<td>2.5 ± 0.5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>starch-free R2A</td>
<td>4.8 ± 0.4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.5% agar R2A</td>
<td>12.3 ± 5.2</td>
<td>-</td>
</tr>
<tr>
<td>LW0508</td>
<td>R2A</td>
<td>1.7 ± 0.4</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>starch-free R2A</td>
<td>4.7 ± 4.1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.5% agar R2A</td>
<td>3.0 ± 0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

The results for LW0504 are shown in Table 3. No clear pattern could be observed for the intensity of growth reduction when different media compositions were used, suggesting that individual competitor strains had their own unique effects on the growth of LW0504. In all cases, though, growth of LW0504 was reduced by at least nearly double (1.7) when cultured with other strains. Despite a clear lack of pattern, the media composition used clearly influenced the competitive ability of LW0504, albeit not in a predictable way. For instance, when LW0501, LW0506 and LW0507 were the co-culture strains, 0.5% agar R2A was found to be highly inimical to growth of LW0504 in the presence of the other strains. When strains LW0502, LW0503 and LW0505 were the co-culture strains, R2A agar was found to be slightly more inhibitory to the competitive ability of LW0504 compared to the other two media compositions. Figure 11 shows these differences.
Figure 11: Growth reduction comparison when normal R2A and 0.5% agar R2A are used. The top row shows growth on R2A with 1.5% agar; the bottom row R2A with 0.5% agar. Each column contains the same co-culture strains—i.e. Figure 4-15A and E contain LW0501, B and F contain LW0507, C and G contain LW0502, and D and H contain LW0505.

Despite not being clearly labelled, LW0504 can be identified in all the photos as the dark orange-red colonies. The first two panels, Figure 11A and B, represent growth of LW0504 with strains LW0501 and LW0507 on R2A agar; below each of these is the respective result on 0.5% strength agar. As can be seen in these photos, growth is clearly inhibited by the presence of competitor strains to a greater extent in the 0.5% strength agar. The competitor strains also seem to produce slightly larger colonies in the 0.5% strength agar, although this could simply be a case of uneven inoculation. In panels C and D, co-cultures with LW0502 and LW0505 on R2A are shown, and below these photos, the respective growth on 0.5% agar is shown. Here, the opposite effect is observed, with LW0504 cultures grown on R2A experiencing a greater growth reduction in the presence of the co-culture strains. Since growth reduction was calculated as the relative difference in size between colonies at the edge of the plate and colonies in the middle, absolute colony size is not taken into account. In Fig. 11H, growth of LW0505 completely dominated the agar plate, resulting in colonies of LW0504 in the middle and at the edge being equally affected. Thus, even though Table 3 suggests that growth in R2A resulted in a slightly larger growth reduction for LW0504, this is clearly not the case. 0.5% strength agar allows for the spreading of LW0505 through the agar, as well as spreading around the edge of the plate, inhibiting LW0504 to the same extent everywhere.
Also presented in Table 3 is whether strain LW0504 showed evidence of twitching motility under the different conditions tested. Interestingly, in most cases, co-culturing on R2A resulted in growth in the interstitial space between the agar and petri dish. Photos of these results are displayed in Figure 12.

Figure 12: Co-cultures with LW0504 and co-isolated strains on R2A. Panel A shows growth with LW0501; B – LW0502; C – LW0503; D – LW0505; F – LW0506; G – LW0507 and H – LW0508. E and I show close ups of growth with LW0501 and LW0503, respectively.

Depending on the co-culture strain, the area of growth underneath the agar changed. In the case of LW0507, shown in Fig. 12G, LW0504 did not show clear evidence of twitching motility. This was the only co-culture strain which did not induce this behaviour in a clearly observable way. Fig. 12E and I show close-ups of growth with LW0501 and LW0503, respectively, wherein the growth underneath the agar was especially clear. Although not measured, the area of growth under the agar was deemed to be strong if it encompassed at least twice the area as the colonies on the surface of the agar. Weak growth was therefore designated as clear growth under the agar which did not reach twice the size of the colonies on the surface. Thus, growth in Fig. 12A, C, D, F and H was said to be strong. Interestingly, all colonies showing strong growth underneath the agar when R2A was used showed weaker growth when starch-free R2A was used. The area of growth potentially resulting from twitching motility seemed to decrease, or become inhibited entirely, when starch-free R2A was used, and when 0.5% strength agar was used, no growth underneath the agar was observed in any case.
4.2.3 Biofilm formation test

The biofilm formation assay, the results of which are shown in Figure 13, did not indicate that strains LW0504, JF1031 or H16F320 could form biofilms under the conditions tested. The average OD values for the three strains were 0.84, 0.63 and 0.33, respectively. T-tests indicated that the difference between optical density for LW0504 and H16F320 was statistically significant (p = 0.00096). The same can be said for H16F320 and JF1031 (p = 0.02629), but not for LW0504 and JF1031. H16F320 was among the weakest adherers tested, suggesting that this strain is incapable of forming biofilms on polystyrene. In contrast, cells of LW0504 adhered to their wells to some extent, but it is unclear from these results whether this strain could form a mature biofilm. Along with LW0502, it is the strongest candidate among strains of the LW05 group. However, none of these strains seem likely to have formed a biofilm when compared to strain JF1023, which exhibits the most consistently high OD values, over twice as high as LW0504. Of all the strains tested, JF1023 appears to be the only one capable of forming a biofilm under the conditions tested. T-tests showed that in all but two cases, the OD difference between JF1023 and the other strains was statistically significant (p < 0.05). Strains JF1034 and H16F314 showed spikes in OD in one or two wells, but in other wells, the OD remained among the lowest, suggesting contamination.

![Figure 13: Visualisation of optical density readings from each well of the Bioscreen plate. The strains corresponding to each of the 5 replicate wells are shown, as well as the average value of absorbance from the 5 replicates for that strain. Darker colours represent greater absorbance, with the darkest well corresponding to an OD of 4.56 and the lightest well corresponding to an OD of 0.100.](image)
5 Discussion

5.1 Biotechnological significance of *Hymenobacter* sp.

Members of the genus *Hymenobacter* are often isolated from environments subjected to fluctuating ecological limitations, including low nutrient levels, freeze-thaw cycles, radiation and low water activity (Collins and Margesin, 2019). Of these abiotic stressors, *Hymenobacter* isolates are most often characterised with respect to their common resistance to UV radiation, most likely due to their frequent co-occurrence with photosynthetic snow algae and lichens. Radiation resistance is of great biotechnological significance; in therapeutics, extremophiles have enormous potential for production of novel anticancer drugs, antioxidants, sunscreens, and other clinically important products (Gabani and Singh, 2013). In place of expensive silicon-based solar cells in renewable energy generation, intense research into third-generation photovoltaic systems has been conducted in the last two decades. This includes dye-sensitized solar cells (DSSCs), which utilises pigment molecules to convert light energy into electricity in a process similar to natural photosynthesis. The red/orange pigments produced by *Hymenobacter* sp., along with their polysaccharide production has implicated them as promising candidates for improving conversion efficiency of DSSCs (Maddah, Berry and Behura, 2020). Radiation-resistant microorganisms have also been implicated in bioremediation of soils contaminated with nuclear waste (Gabani and Singh, 2013).

The extremophilic adaptations exhibited by *Hymenobacter* sp. also include resistance to low temperatures. Enzymes produced by cold-adapted microbes have received increasing attention in recent years: cold-active enzymes have high specific activities at low temperatures, and are inactivated easily by increasing the temperature, making them useful for industrial, agricultural, and medical applications (Lee et al., 2012). In a recent study on *Hymenobacter* sp. strain UV11, it was found to have enormous potential for production of extracellular hydrolytic enzymes with broad biotechnological uses (Marizcurrena et al., 2019). *Hymenobacter* sp. CKS3 has been shown to have huge enzymatic potential for waste bread hydrolysis in bioethanol production (Mihajlovski, Rajilić-Stojanović and Dimitrijević-Branković, 2020).
Clearly, the study of *Hymenobacter* sp. is of great value for biotechnological applications, and research into representatives of the genus should be widespread to account for further potential valuable features. While hymenobacters are often initially isolated and subsequently investigated for production of cold-active hydrolytic enzymes or radiation-resistance, reports in the literature of more extensive work on their characterisation is lacking. Their biotechnological significance seems to stem mainly from their extremophilic adaptations: this study was aimed at investigating another common abiotic stressor, in the form of osmotic shock. This stressor frequently arises when sub-zero temperatures induce ice formation, which concomitantly provokes the exclusion of solutes and removal of available liquid water from the microbial extracellular space (Collins and Margesin, 2019). Osmotic stress is usually investigated with respect to food microbiology: research into the ability of common pathogenic and food spoilage bacteria to proliferate in food is invaluable in the design of preservation methods and general food safety. *Hymenobacter* sp. are environmental bacteria not commonly found in food (although Niu et al. (2020) recently reported the presence of *Hymenobacter* in fried chili), but research into osmotic stress tolerance is still pertinent for numerous applications. Broadening our understanding of how extremophiles respond to the stressors they face in their natural environment will give valuable insight into the molecular mechanisms that differentiate them from less tolerant microorganisms.

Osmotic stress tolerance can also have industrial relevance: it is important to understand how bacteria behave and respond to conditions that occur during industrial processes. When selecting strains for industry-scale fermentations or mass-production of enzymes, for instance, it is important that they do not become inhibited by the high concentration of substrates or products in the fermentation medium (high osmotic pressure) (Szymanowska-Powałowska, 2015). In environmental microbiology, bioremediation of wastewater from aquaculture and fishing industries, chemical factories, oil refineries, tanneries, and the food, beverage and textile industries is complicated by high salinity. Microorganisms adapted to tolerating osmotic stress are better equipped to survive these conditions (Vyrides and Stuckey, 2017). Gerday et al. (2000) suggest that psychrophilic enzymes may be better equipped to catalyse reactions under low water conditions due to their flexibility, important in the commercial synthesis of compounds obtained from substrates with poor solubility in aqueous media. Thus, tolerance to osmotic stress has significant applications in numerous fields: based on the environments from
which the *Hymenobacter* isolates in the present study were sampled from, it was hypothesised that they would possess intrinsic mechanisms conferring tolerance to osmotic stress. Interestingly, this was not the case.

### 5.2 Osmotic stress tolerance

#### 5.2.1 *Hymenobacter* strains H16F320, JF1031 and LW0504 are sensitive to osmotic stress

Based on the conditions used in the present study, the *Hymenobacter* strains appear to be unequivocally intolerant of osmotic stress. The results of both growth experiments indicate reproducibility, in that growth at lowered water activity was not observed in either case in the time frame studied, even with the lowest humectant concentrations. The exception to this is when glycerol was used to adjust the $a_w$ to 0.975 in the second run of the experiment: here, JF1031 was able to grow at the same rate as under standard conditions, after experiencing an increased lag time. The lag phase is an adjustment period where bacterial cells modify themselves in order to calibrate to a new environment, resulting in a time period before exponential growth commences wherein no growth occurs (Swinnen *et al.*, 2004). Bacteria that have been injured by stress factors such as osmotic shock require more time to exit the lag phase (Bertrand, 2019), since there are more adjustments to be made in order to adapt to unfavourable conditions. Zhou *et al.* (2011) studied the effects of osmotic stress on the lag phase of *Salmonella enterica*: when grown in a minimal medium, *S. enterica* exhibited an increasing lag time as the NaCl concentration of the medium increased, with a dramatic increase in lag time when the water activity was close to the growth/no-growth interface of the bacterium. Interestingly, when the nutrient-rich LB medium was used, the lag phase did not change with increasing salt; instead, the specific growth rate decreased (Zhou *et al.*, 2011). The observations made from Fig. 4-6F are more in line with the former scenario, where the growth rate of JF1031 was unaffected by glycerol, but the lag phase time increased by about 50 hours. When glycerol was used to adjust the $a_w$ to 0.962, no growth occurred for JF1031, suggesting that the minimum water activity for growth when glycerol is used as the humectant is somewhere between these two activities. In the first run of the experiment, no growth was observed for JF1031 at $a_w$ 0.975 with glycerol: this contradicting result is interesting, and warrants further investigation.
Similar to the hymenobacters, the closely-related *Dyadobacter* strains MAS034 and MAS040 displayed great sensitivity to osmotic stress. No growth that could be attributed to the strains in question occurred when NaCl and sucrose were used to lower the water activity: with glycerol, once again, limited growth was observed at $a_w$ 0.975 for MAS034; MAS040 showed better growth at this water activity than under standard conditions, but since only one of three replicates provoked this observation, it was not considered significant. The slight osmotolerance exhibited when glycerol is used to control the $a_w$ can be attributed to the specific solute effects that arise due to the permeable nature of this solute. Glycerol does not initiate the same osmoregulatory response as non-permeant solutes such as sodium chloride and sucrose (Tapia *et al*., 2007), and can even protect microbes against chaotrope-induced stresses at low concentrations; at inhibitory concentrations, however, this solute itself can act as a chaotrophic agent (Stevenson *et al*., 2015). While glycerol is commonly reported to be less inhibitory than NaCl to non-halophilic pathogenic bacteria (Tapia *et al*., 2007), the studies carried out to determine the limits of microbial solute tolerance most frequently focus on copiotrophic microbes. As such, there is less data available about the water-activity limits for slow-growing species isolated from oligotrophic environments (Stevenson *et al*., 2015). From the results of this experiment, the *Hymenobacter* species appear to have high water activity requirements for growth, and glycerol is less inhibitory than NaCl and sucrose, at least for strain JF1031. The opposite effect was observed for strain AR0120, the halophilic *Salinicoccus* sp., where NaCl allowed for a higher growth potential (AUC) than the other humectants at all water activities tested. This is in line with the fact that the halotolerant *Staphylococcus aureus* has a lower minimum water activity for growth when NaCl is used to lower the $a_w$ than when glycerol is used (Vilhelmsen and Miller, 2002). Previous work on the *Hymenobacter* strains used in this study revealed that they could not grow in the presence of over 1% NaCl in the growth medium: in this study, the highest water activity level with NaCl as the humectant corresponds to a 0.6 molar solution, or about 3.5% (w/v). It is therefore not surprising that at water activities controlled by NaCl, no growth occurred: however, the use of sucrose and glycerol as alternative humectants did not significantly increase the osmotolerance of the strains. As such, it can be reasonably confidently asserted that the effects of low water activity and osmotic stress (rather than, for instance, the ionic strength of NaCl) are inhibitory for *Hymenobacter* strains H16F320, JF1031 and LW0504.
5.2.2 The lack of osmotic stress tolerance does not reflect the environmental context from which the strains were isolated

The lack of osmotic stress tolerance exhibited by the strains is perhaps a rather surprising result. The harsh environments from which the isolates were sampled likely experience cycles of stressors including osmotic stress: their total lack of tolerance would imply their inability to persevere in such hostile environments. Based on their other features, the strains would also perhaps have been expected to better tolerate the relatively moderate osmotic stress imposed on them in this study. For instance, studies have shown that radiation resistance has a strong correlation with desiccation resistance, and indeed some researchers hypothesise that radiation resistance is an incidental consequence of the ROS scavenging system that protects cells against desiccation (Yu et al., 2013; Musoliva et al., 2015). Of course, osmotic and desiccation stress are very different: while osmotic stress refers to an abundance of solutes in the surrounding environment, desiccation stress results from the lack of water (Vriezen, de Bruijn and Nüsslein, 2007). Despite this, there exists evidence of a relationship between these two water-related stressors, and an overlap in the stress response mechanisms they activate: for instance, Chen and Alexander (1973) showed that a higher percentage of desiccation-tolerant than desiccation-sensitive bacteria were able to grow in media with high salt concentrations. Hingston, Piercey and Truelstrup (2015) identified 30 desiccation-associated loci in the genome of L. monocytogenes, and concluded that 19 of these genes are likely to have roles in osmotic stress adaptation as well. To validate this potential connection and further explore ecological stressor tolerance, future work should determine the survivability of the Hymenobacter strains when exposed to desiccation: perhaps the strains exhibit xerotolerance, even in the absence of osmotolerance. The overproduction of EPS common to members of this genus may be indicative of their capacity for desiccation tolerance, akin to the radiation- and desiccation-resistant cyanobacterium Nostoc commune (Helm et al., 2000).

The finding that the Icelandic Hymenobacter isolates appear to be almost totally intolerant of osmotic stress is almost more interesting than the alternative, and future studies could turn towards investigating how they are able to persist in conditions that can be inimical to their growth. It should be noted that bacterial stress responses can be shaped temporally by their ecological context; it is possible that environmental bacteria ‘anticipate’ a succession of stressors in order to minimize energy expenditure and therefore enact regulatory mechanisms.
in a chain that reflects the order in which the stressors appear. For instance, osmotic stress in the natural environment could be preceded by a period of sub-zero temperatures wherein water freezes, resulting in the gradual increase of solute concentrations. It is therefore conceivable that the strains may have evolved a specific response to osmotic stress that is only activated upon freezing temperatures - an example of cross-tolerance that deserves further study. It should also be stressed that the present study subjected the cells to a sudden hyperosmotic shock upon inoculation, which may be an unlikely event in the natural context: the onset of osmotic stress may be gradual, and stress response systems may therefore have evolved to activate more slowly. Also relevant is the larger community context: bacteria live in dense multi-species communities, and social interactions between community members may determine outcomes of ecological stress in ways that axenic studies are unable to appreciate. For instance, the *Hymenobacter* strains could overcome their intolerance to osmotic forces by relying on other members of the multi-species assemblies within which they reside. Yao et al. (2020) recently showed that co-culturing *Tetragenococcus halophilus* with *Zygosaccharomyces rouxii* improved the response of the latter to salt stress by slowing down the depletion of amino acids. Stress resistance mechanisms are energetically expensive: synthesis of a single molecule of the compatible solute ectoine requires the expenditure of around 40 high-energy bonds, while transporting one molecule of ectoine into the cell requires hydrolysis of just two ATP molecules (Bremer and Krämer, 2019). It is therefore conceivable that producer bacteria in the community secrete compatible solutes as public goods which other bacteria can utilise to survive periods of osmotic stress (Bremer and Krämer, 2019). A good first step to explore this idea would be to repeat the growth experiment, but supply the medium with various exogenous compatible solutes to determine whether the *Hymenobacter* sp. can import them into their cytoplasm and benefit from their osmoprotective qualities, despite not being able to synthesise them on their own. Another measure that will help to validate these ideas is to sequence the genome of the *Hymenobacter* strains: the absence or presence of various genes will paint a clearer picture of the physiological tools available to respond to environmental stressors. For instance, many microorganisms that are unable to synthesise the compatible solute glycine betaine have been found to express GB transport systems that can uptake GB from the growth medium (Vyrides and Stuckey, 2017). *Hymenobacter* sp. strain IS2118 was found to have 7 osmotic stress-responsive genes encoded in its genome; strain UV11 has genes...
for carbon-based compound storage, implicated in starvation, desiccation and osmotic stress endurance, as well as genes involved in the production of the compatible solute trehalose (Koo et al., 2014; Marizcurrena et al., 2019). It would be interesting to explore whether strains H16F320, JF1031, LW0504 and FN1603 contain similar genomic features.

5.2.3 Growth considerations under standard conditions

One interesting discussion point and potential shortcoming of the growth experiment is the apparent lack of reproducibility between the two runs of the experiment. Since no growth under conditions of lowered water activity occurred for the hymenobacters (with the exception of one case), this is solely in referral to growth under standard conditions, which were defined as room temperature (23°C) and pressure, and R2A liquid broth as the growth medium. Interestingly, the growth curves for the three strains were all significantly different between the two runs of the experiment. Analysis of the growth parameter data generated using Growthcurver determined that, depending on the strain, variations in $r$, $K$, AUC and Tgen values were statistically significant even though growth conditions were the same. Several explanations could be postulated for this result. The first is the difference in water activity measured for the growth medium: for the first experiment, this was determined to be 0.989, while in the second run it was measured as 1.003. The liquid media was prepared separately for each experiment, so it isn’t inconceivable that there could be small differences in the observed water activity—but a difference of 0.014 seems improbable. Furthermore, since pure water has a water activity of 1.000, the value from the second run is impossibly high: it is therefore likely that the chilled mirror hygrometer used to measure the water activity was incorrectly calibrated during the second experiment. The device determines water activity via a dewpoint sensor which measures the point at which the water activity of the sample and the relative humidity of the air are in equilibrium. The sample is placed in a chamber containing a mirror and a condensation detector; the presence of condensed water on the mirror is detected as a reduction in the mirror’s reflection (Tuan, 2014). The mirror requires occasional cleaning to retain its function, and it is possible that in this case the mirror needed to be cleaned: this calls into question the reliability of the water activity measurements conducted for the second run of the experiment.
The growth phase of the precultures used for the growth experiments could also be of significance. Vilhelmsson and Miller (2002) showed that *S. aureus* cultures harvested while still in the exponential phase displayed significantly longer lag times, lower growth rates and even higher growth-inhibitory *a*<sub>w</sub> than those derived from the stationary phase. This could be the explanation for the significant differences in growth parameters exhibited by the strains; the cells at the start of each experiment may have been harvested from pre-cultures in different phases of growth. Although in both cases the pre-cultures were grown for 6 days, this does not necessarily mean they were in the same growth phase at the time of harvesting. Augustin *et al.* (2004) showed that with *L. monocytogenes*, the lag time is extended under conditions of starvation when the inoculum size is very small. Since the inoculum size in this study was not standardised owing to the EPS production of *Hymenobacter* in liquid media, it is conceivable that the lag times and therefore growth phases of pre-cultures between the experiments may have differed.

A third observation about the nature of growth of the hymenobacters used in this study deserves mention. Namely, it was noted that upon repeated sub-culturing, the strains sometimes, rather unpredictably, began to lose viability. Although there is no data to support this, the growth of the strains was greatly diminished in some cases after sub-culturing them a few times, resulting in the need to re-plate them from their original stocks, at which point they grew normally. Strain FN1603 was able to be cultured in previous studies, although the sub-culture-induced loss of viability was also strongly observed for that strain; in the present study, repeated attempts to culture strain FN1603 were ineffective. Multiple studies have remarked on an observed decrease in virulence when strains of certain pathogens are sub-cultured for many generations (Molina-Torres *et al.*, 2010): however, the dramatic loss of viability after only 2-3 successive sub-culturing steps is not as commonly reported. After 31 successive sub-cultures, the fungus *Neocallimastix hurleyensis* did not exhibit a change in survivability, but depending on the carbon source used for cultivation, there was a significant decrease in various enzymatic activities (Ekinci, Ozkose and Akyol, 2006). It would be worth investigating whether growth on differing carbon sources might affect survivability of the *Hymenobacter* strains after several successive sub-cultures. For the strains isolated from lichens (JF1031 and FN1603), it is possible that the minimal R2A media may not adequately reproduce the complex nutritive conditions of lichen thalli (Biosca *et al.*, 2016). Continued growth on this oligotrophic media
may therefore constitute stress, and growth may turn to maintenance after a few generations. Smith and Bidochka (1998) noticed a decrease in growth rate of *E. coli* after sub-culturing colonies a few times in minimal media, likely as a starvation-induced stress response. Prolonged nutrient starvation often results in dormancy, or alternatively the recently described ‘oligotrophic growth state’, where non-sporulating *Bacillus subtilis* cells were observed to grow more than 100 times more slowly than normal exponentially growing cells (Gray et al., 2019). Another possibility is the viable but nonculturable state, where cells under stress retain active metabolism but are incapable of growth on a medium normally supportive of their growth (Oliver, 1993). The lack of certain nutrients may therefore be instrumental in the loss of the ability of the strains to grow on media on which they are routinely cultured.

5.3 Competitive strategies and interspecific interactions

5.3.1 Growth of strains H16F320 and LW0504 is negatively affected when they are cultured with co-isolated species

The interaction assays performed by co-culturing the *Hymenobacter* strains with their respective co-occurring isolates suggest that both H16F320 and LW0504 perform poorly in competition for space and resources. In all cases, the growth of these two strains was reduced, sometimes dramatically, as they got closer to their competitors. The experiment is designed as a simple method of assaying interactions between two species, whether they be antagonistic or synergistic. With the strains investigated, synergistic interactions did not occur; this is not a strange result, but bacterial growth has sometimes been shown to be aided by the presence of another species; in mixed-species biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum*, the cell numbers of the former species were increased compared with a single species biofilm (Van der Veen and Abee, 2011). Antagonism is the much more likely scenario, especially since cooperation usually results from specific interactions that are only applicable to certain situations. For instance, culturing *S. cerevisiae* with *Lactobacillus rhamnosus* enhances the survivability of the former at pH 2.5 and 3.0, but this protective effect is not observed once the pH is increased to 4.0 (Lim, Toh and Liu, 2015). Competition, on the other hand, does not need to result from reactive interactions, as in interference competition, but can simply be a by-product of both species competing for the same limited nutrient, as in
exploitative competition. The fact that the co-culture strains were isolated from the same samples and therefore likely have similar resource requirements would suggest that, according to the habitat filtering principle, competition between the two is a likely outcome. The results support this fact, showing how the hymenobacters are consistently outcompeted: this raises the question of how they are able to maintain a viable population in their natural environment.

Of course, if the environment from which the species were isolated contains multiple resources or niches, selection for competition is reduced and the species may be able to coexist simply by using different resources (Ghoul and Mitri, 2016). One of the most common results of co-culturing bacteria is a shift in their enzymatic profiles. Co-culturing *Bifidobacterium longum* and *Bifidobacterium breve* resulted in an increase in activity of enzymes involved in the degradation of various complex carbohydrates for both species (Ruiz *et al.*, 2009). The observed lack of competitive ability of the hymenobacters may therefore reflect the simple nutrient composition of the medium: while they might have a lower substrate specificity or rate of uptake for glucose, culturing the competitors on different carbon sources may produce different results. Strain LW0504, which was outcompeted by all 7 co-culture strains, was isolated from barren mountaintop soil, a habitat characterised by diverse complex carbohydrates. Larsbrink and McKee (2019), in their review on soil *Bacteroidetes* sp., suggested that a common competitive strategy for members of this phylum is to produce specific hydrolytic enzymes only when the appropriate substrate is available; they go on to say that species that secrete extracellular slime may do so to sequester these enzymes and retain the resulting released nutrients. Previous work on the Icelandic *Hymenobacter* strains showed that they produce various extracellular enzymes, as well as large amounts of EPS: further work should attempt to explore these features within the context of competition.

Although little can be decisively concluded due to the fact that the assay performed is simplistic, and the results should therefore not be used to make sweeping inferences, it can be said that under the conditions tested, growth of the *Hymenobacter* strains is negatively affected when cultured with co-isolated strains. This is likely due to exploitative competition, where the competing strains simply outgrew the hymenobacters by virtue of their faster growth rates and shorter lag times. Early and rapid growth can be expected to confer distinct advantages to competitive ability, since the direct utilisation of limiting nutrients lowers the pool of available nutrients for the slower growing strain. Under competition with glucose as the
sole carbon source, *Halomonas* HL-48 outcompeted *Marinobacter* HL-58 with very little change in its transcriptional profile or specific growth rate, simply because the former is a faster growing species. In contrast, *Marinobacter* was very sensitive to glucose competition, drastically shifting gene expression in an attempt to respond, and ultimately suffering a 20% reduction in its specific growth rate (Khan et al., 2018). It is of course also possible that interference competition played a part in the reduced growth of the *Hymenobacter* sp. when in close proximity with the co-culture strains; Ruiz et al. (2009) suggest that the 42% reduction in specific growth rate observed for *B. breve* when co-cultured with *B. longum* may have been due to the production of inhibitory substances, as *B. longum* is able to produce antimicrobial compounds. However, the authors note that their results may combine effects caused by nutrient starvation, production of antimicrobials, etc., on specific growth rates, and therefore should not be interpreted conclusively. Due to the simplicity of the assay in the current study, a similar caution should be exercised, before further experiments can reliably reveal the nature of this competitive outcome.

5.3.2 Culture-dependent and culture-independent research on microbial community interactions

One of the limitations of the assay is its simplicity, making it hard to interpret the results, but simple preliminary assays are important to reveal interesting features that are worth studying more. The fact that every co-culture strain negatively influenced growth of the *Hymenobacter* sp., for instance, is interesting and deserves further investigation. However, this interesting result could itself be a product of the limitations of the assay, or rather, the limitations of culture-dependent studies on bacterial interactions as a whole. Consider a complex bacterial community; the interplay between species can be viewed as a network of cooperative and competitive interactions. Some individuals who form cooperative relationships with other species may play a small crucial role in a larger cooperative chain: an individual could, for instance, specialise in the degradation of a particular nutrient. Another individual, who may not possess the abilities of the former, could benefit from this degradation. If the first species were to disappear, the second may not be able to survive in the environment due to the loss of this essential nutrient. Thus, bacteria that are auxotrophic for some critical function may be obligate co-operators, relying on the presence of other species capable of performing a particular function: this has been postulated as a major reason for why environmental bacteria are
notoriously unculturable (Pinto et al., 2019). Since strains that have been sampled from the environment are always isolated first to produce pure cultures that can be researched in the laboratory, the loss of many co-dependent strains is inevitable. Therefore, synergistic interactions are likely underrepresented in these assays, as bacteria that would benefit the most from such cooperation would not have been isolated in the first place.

Co-cultures are very simple synthetic ecosystems, allowing us to study natural communities with a selected set of species and specific conditions. They are easy to control due to their reduced complexity: therefore, they provide a powerful way to test ecological theories and begin to understand the underpinnings of bacterial interactions (Heyse et al., 2019). A problem with this type of study is that species may meet in the laboratory that would never meet in reality; spatial organisation of strains in their original niche is often destroyed through sampling. The co-isolated strains in this study may actually never interact in reality, due to living in separate clonal patches that are millimetres away from one another, a potentially significant distance in the eyes of microorganisms (Ghoul and Mitri, 2016). These simple studies therefore run the risk of failing to capture the bigger picture; complementing them with other methods should allow for higher-resolution data with more meaningful interpretations. While co-culturing different strain combinations in the laboratory represents a bottom-up approach for studying community interactions, top-down approaches are equally valuable. These include genomic, transcriptomic and metabolomic methods (Ghoul and Mitri, 2016). These culture-independent methods can capture a much better representation of complex communities owing to their high-throughput nature: millions of reads can be produced from a single sample, allowing for the accurate prediction of microbial relationships. This is based on the premise that distribution patterns are likely due to ecological reasons: for instance, when two species co-occur over many samples, a positive relationship is assumed, and conversely, a negative relationship is predicted when two species show mutual exclusion across multiple samples (Faus and Raes, 2012). With complex co-occurrence networks, you can begin to form ideas about specific pairwise relationships that make up the community, as well as its organisation as a whole. Of course, relating these ideas back to actual ecological interactions is not easy; positive relationships can arise due to co-aggregation in biofilms, niche overlap, co-colonization, or many other reasons. Negative relationships in turn can be shaped by many forces, competition being only one possibility (Faus and Raes, 2012). In order to correctly
interpret these associations and relationships, experimental validation is required, which is why combining these culture-independent methods with culture-dependent methods is especially promising. For instance, the results of this study would be easier to interpret if data was available on the co-occurrence of the co-culture strains: do the *Hymenobacter* strains occur at a lower rate in samples containing these fierce competitors, or is the experimental design simply inimical to their competitive ability?

5.3.3 Strains H16F320 and LW0504 appear to induce twitching motility under certain conditions

The apparent observed induction of motility in strains H16F320 and LW0504 upon inoculation with certain co-isolated species is incredibly interesting, since members of the species *Hymenobacter* are typically described as nonmotile. Despite this, many representatives of the genus form colonies with spreading edges, which is suggestive of gliding motility. Furthermore, a few members were originally referred to using the genus name “*Taxeobacter*”, which was reserved for gliding bacteria (McBride *et al.*, 2014). Gliding motility is a common characteristic of members of the phylum *Bacteroidetes*, and has been most extensively characterised for *Flavobacterium johnsoniae*, which can be considered a model organism for the study of various physiological features common to members of this phylum (Larsbrink and McKee, 2019). Many proteins, initially identified in *F. johnsoniae* as being required for gliding motility, have since been attributed to function in protein secretion as well (Kulkarni *et al.*, 2017). These proteins are involved in a secretion system found in most *Bacteroidetes*, but not in any other phyla, known as the Type IX Secretion System (T9SS) (McBride, 2019). The role of T9SS is to allow for the transportation of proteins necessary for things such as virulence, nutrition, and importantly, gliding motility (Lasica *et al.*, 2017). Proteins that are secreted across the cell membrane have an amino-terminal signal peptide, targeting them to the standard Sec export system; additionally, these proteins contain conserved carboxy-terminal domains (CTDs) that direct them to the T9SS (Kulkarni *et al.*, 2017). A recent comparative genomic study found that, of the complete *Hymenobacter* genomes that have been published, all of them contain dozens of proteins with conserved CTDs, as well as genes encoding T9SS components (Johnston, 2019). This suggests that the secretion system may be conserved in this genus, and since gliding motility and T9SS are interlinked (McBride, 2019), could explain the gliding motility sometimes observed for members of the genus.
Interestingly, however, the motility observed in the present co-culture assays had characteristics more indicative of twitching motility, rather than gliding motility. Active twitching motility often manifests at the interstitial surface between agar and the plastic (or glass) petri dish, resulting in large twitching zones underneath the agar. This is because of the smoothness of the surface; the lack of twitching motility on the surface of the agar can be ascribed to inhibition via air-drying, resulting in irregularities which hinder movement. These irregularities do not occur when the agar is solidified against a smooth solid surface, such as the bottom of the petri dish, allowing for more rapid expansion (Mattick, 2002). On a microscopic level, twitching motility can be observed as an outward movement from the colony centre of rafts or spearhead-like clusters of aggregated cells. The cells in these rafts are highly aligned in tight cell-cell contact, and as they advance, groups of cells break up into smaller aggregates, moving off in different directions and cross-connecting with other groups (Mattick, 2002). This eventually leads to a lattice-like network of trails; interestingly, similar trails and cell reversals are also characteristic of social gliding motility in *Myxococcus xanthus* (Harshey, 2003). Similarly, gliding motility of *Synechocystis* follows the same process; while twitching is generally used to describe discontinuous cell movements and gliding usually connotes smooth movements, McBride (2001) suggests that distinction between the two is a subjective matter. Indeed, twitching motility and social gliding motility exhibited by myxobacteria have been found to be essentially equivalent (Semmler, Whitchurch and Mattick, 1999). The mechanism of both these forms of motility involves type IV pili, the active extension and retraction of which provides the driving force for movement; furthermore, both types of motility are primarily social activities, requiring direct cell-cell contact and the formation of cell aggregates (Harshey, 2003). *M. xanthus* also exhibits a different form of motility known as adventurous motility: while the advancing edges of bacteria utilising this form of motion look similar to twitching and social gliding, this form of gliding occurs without pili (Harshey, 2003). It is this form of gliding employed by most *Bacteroidetes*, as motility mediated by the T9SS does not require pili. While phenotypically, the motility observed in the current study appears to be of the twitching variety, this should be studied in-depth to confirm the true mechanism of movement.
5.3.4 The presence of co-isolated strains can induce motility in H16F320 and LW0504

The twitching motility observed for H16F320 and LW0504 was only induced under certain conditions, depending on the media composition as well as the identity of the co-culture strain. Accordingly, H16F315 but not H16F314 induced motility in H16F320 when co-cultured on standard R2A; all co-culture strains except for LW0507 were found to induce twitching motility in LW0504. This phenomenon, while unexpected, is certainly not unprecedented. Many researchers have reported motility as an induced phenotype resulting from interspecific interactions: *Streptomyces venezualae*, previously thought to lead a completely static lifestyle, was found to initiate ‘exploratory’ motility when cultured in the presence of some species of yeast (Jones *et al*., 2017). *Xanthomonas perforans*, a nonmotile bacterium, induces surface motility in proficient motile bacteria, allowing the xanthomonad to ‘hitchhike’ on top of the motile swarming rafts (Hagai *et al*., 2013). The increasing number of instances in which interaction-associated motility is reported means that the concept of motility in the ecological context needs to be reconsidered; investigations into the prominence of motility and its functional role in microbial communities can not be ascertained through studying individual species in axenic laboratory cultures (McCully *et al*., 2019).

The twitching motility phenotype was only activated in some co-cultures; interestingly, in almost all cases where motility was induced in strains H16F320 and LW0504, the co-culture strain exhibited a similar level of motility. The exceptions to this are strain LW0506, which showed very limited twitching compared to LW0504, and strain LW0507, which did not induce twitching in LW0504. In order to uncover any patterns to this induced behaviour, further experimentation is required: only 2 co-isolated strains were tested for H16F320, for instance. Interestingly, McCully *et al*. (2019) found that co-isolation of strains was not an important factor for their ability to engage in social motility; conversely, phylogenetic relatedness was a more accurate predictor of interaction. Studying co-isolated species is likely to result in a closer representation of the actual ecological interactions of *Hymenobacter*; it would be interesting to explore whether the differences in social behaviour could be a function of the phylogenetic relatedness of the two co-isolates, i.e. whether the species which induced motility in one another are more closely related than the others. As only a few of the co-isolated strains have had their 16S genome sequenced, little can be extrapolated from their relatedness to the
Hymenobacter isolates. For instance, LW0507 shares the greatest 16S sequence similarity with Arthrobacter humicola, a member of the Micrococaceae family, while LW0502 shares the most similarity with Acaricomes phytoseiuli, also a member of the Micrococaceae family. LW0507 failed to induce motility in Hymenobacter sp. LW0504, whereas LW0502 did not. Strain H16F315 also belongs to a species of the genus Arthrobacter, but this species did induce motility in H16F320: strain H16F314, which did not induce motility, does not have a sequenced 16S gene.

5.3.5 Agar concentration and nutrient availability affect the motility of H16F320 and LW0504

As well as the identity of the co-isolated strain, the media composition was found to affect the induction and extent of motile activity. The agar concentration was found to be especially significant: on standard R2A, which contains an agar concentration of 1.5%, motility was commonly exhibited. Decreasing the agar concentration to 0.5% while keeping all other parameters constant resulted in a complete inhibition of the twitching motility phenotype in strains H16F320 and LW0504. The importance of agar concentration on the morphology of motile bacteria has been well documented: for instance, Mitchell and Wimpenny (1997) suggest that the agar acts as a ‘net’ with variable pore sizes based on its concentration, and that there is a certain critical threshold above which submerged motile cells are not able to move. Below this critical concentration of 0.65%, motile cells can move away from the central colony, resulting in large, spherical and almost transparent ‘wispy’ structures. This could be the explanation for the drastic change in morphology for strain LW0505 cultured on 0.5% agar, which is below the critical threshold of 0.65%. However, it does not explain the inhibition of twitching motility observed for the Hymenobacter strains and co-isolates alike. A range of environmental factors, including surface conditions, may serve as cues for motility; in the laboratory, this translates to the hardness and moisture content of the surface of the agar. At low or high agar concentrations, bacteria may exhibit alternate motility phenotypes: for instance, P. aeruginosa swarming is induced with 0.4-0.7% agar, while twitching occurs at 1.0% agar (Mattingly et al., 2018). Twitching has been shown to be dependent on the availability of moisture, while social gliding in myxobacteria can work under drier conditions, such as 1.5% agar (Harshey, 2003). Since high agar concentrations retain water better, the firmness of the surface can decrease the surface wetness; however, twitching motility typically occurs in the
film of water between the agar and the petri dish, where the moisture level is higher (Mattingly et al., 2018). It could therefore be the case that the decreased surface firmness is the reason for the inhibition of twitching motility in 0.5% agar.

In oligotrophic environments, bacteria may rely on motility to scavenge nutrients for survival. Simulations have shown that starved cells invoke the strongest motility response under nutrient-poor conditions; therefore, some bacteria expend precious energy for the potential benefit of nutrient acquisition (Du et al., 2020). Soil *Bacteroidetes* use gliding motility to ‘hunt’ for the nutrients they need, which is perhaps the reason why this unique form of motility is intrinsically tied to the system they use to secrete hydrolytic enzymes (Larsbrink and McKee, 2019). LW0504 was isolated from barren mountaintop soil and H16F320 from ice in a lava tube cave: both of these strains would benefit from being able to move around in their respective oligotrophic environments. It would be interesting to compare the results from these two strains with JF1031 and FN1603, both of which were isolated from lichens. Interestingly, while gliding motility of *Bacteroidetes* is used to hunt for nutrients under oligotrophic conditions, exploration via twitching motility is most often dependent on relatively high nutrient concentrations (Harshey, 2003). At high nutrient concentrations, colonies exhibit expeditionary movement over new surfaces. Conversely, under conditions of nutrient depletion, twitching motility is used to the opposite effect: cells are brought together into structures such as biofilms or fruiting bodies as a stress response (Mattick, 2002). Under starvation, production of type IV pili may therefore constitute too high an energy expenditure. By omitting starch from the medium, slight nutrient deprivation was imposed on the co-culture strains: in all cases where twitching motility was not inhibited, it was induced to a much lesser extent than with standard R2A. This is in line with the fact that low nutrient concentrations do not promote outward-expanding motility. By reducing nutrient concentrations further i.e. with 50% or 10% strength R2A, motility may potentially be completely inhibited. The observations that twitching motility requires surface wetness and high nutrients can be supported by the observations of Pinto et al. (2019), who studied arctic snow bacterial communities adapted to oligotrophic lifestyles. As the spring season progressed, temperatures gradually got warmer: this was accompanied by an increase in carbon content as well as wetness. Accordingly, they noticed an increase in the relative abundance of proteins related to motility in bacterial populations within the snow.
Different species of bacteria live in different environments: the regulation of twitching motility is intertwined with the ecological context within which bacteria reside. Thus, different species of bacteria regulate twitching motility in different ways to respond to different stimuli. *P. aeruginosa* and *M. xanthus* initiate motility using a variety of environmental and nutritional signals; in *Synechocystis* sp. PCC 6803, light controls motility and production of type IV pili (Mattick, 2002). Understanding the mechanisms that dictate how bacteria respond to environmental factors is of great significance, especially since these factors often change the nature of interspecific interactions (Khan *et al.*, 2018; McCully *et al.*, 2019). As well as the mechanisms used to regulate twitching motility, it is of equally great significance to discover the motility strategies employed by these bacteria. Surface motility is an energetically expensive endeavour, and it naturally follows that bacteria would not waste precious energy on the freedom of movement unless they have a very good reason for doing so. Diverse motility strategies have been unearthed by research on interspecific interactions of environmental bacteria. Some appear to be cooperative, such as the social co-migration of *Pedobacter* sp. strain V48 and *Pseudomonas fluorescens* Pf0-1, where neither species exhibits motility when cultured on their own (McCully *et al.*, 2019). Many strategies are employed in response to competition, whether they be a means of escaping a stronger competitor, such as *B. subtilis* fleeing from lethal antibiotic-producing *Streptomyces* sp. (Stubbendieck and Straight, 2015), or facing them head on, as with *P. aeruginosa* spreading over and ‘blanketing’ *A. tumefaciens* biofilms (An *et al.*, 2006). The last example highlights another strategy employed by a great number of environmental bacteria facing both competition from other species and stress from environmental factors - the formation of biofilms. Interestingly, type IV pili and twitching motility have been shown to be required to induce biofilm formation for some species of bacteria, including *P. aeruginosa* (Semmler, Whitchurch and Mattick, 1999). Accordingly, biofilm formation for the *Hymenobacter* strains was examined.

The link between motility and biofilm formation is complex: biofilm bacteria are sessile, and so the two strategies are often considered to be diametrically opposed (Verstraeten *et al.*, 2008). The formation of biofilms requires both bacterial self-aggregation and surface adhesion (Du *et al.*, 2020). Twitching motility requires cell-cell contact, as well as cell attachment to
surfaces. It is therefore not surprising that type IV pili have been shown to play a role in surface attachment and biofilm formation in multiple species, including Xylella fastidiosa, Acidovorax avenae and Stenotrophomonas maltophilia (Li et al., 2007; Bahar, Goffer and Burdman, 2009; Huang, Somers and Wong, 2006). Biofilms are believed to be the dominant lifestyle of bacteria in environmental ecosystems, typically comprising diverse multi-species assemblages governed by complex interactions (Ren et al., 2014). Since strains H16F320 and LW0504 appear to be capable of twitching motility under some conditions, they must be able to produce type IV pili. These factors together made it reasonable to assess the ability of the isolates to form biofilms: twitching in S. maltophilia, for instance, is positively correlated with biofilm development in polystyrene microtiter plates (Huang et al., 2006). Interestingly, the Hymenobacter strains do not appear to possess this ability, at least not under the conditions tested. Biofilm formation is often stimulated by conditions such as competing strains or environmental stressors; in this study, the ability of the strains to form biofilms was examined in mono-culture and slightly nutrient-deprived media. It is entirely possible that the hymenobacters do not initiate biofilm formation unless under certain conditions, such as the presence of competitors. Since twitching motility was found to be conditionally induced based on the identity of the co-cultured strain, this too could be the case for biofilm formation, and therefore requires further study. Various strains co-isolated with the Hymenobacter strains were also assessed for their ability to form biofilms; of those tested, JF1023 appears to be the only strain capable of biofilm formation in mono-culture. It would be interesting to see whether co-culturing these strains together alters the results of the present study.

5.3.7 Relationship between microbial community dynamics and ecological traits

It is worth mentioning that along with motility, extracellular polymeric substance production plays an integral role in biofilm formation. The secreted EPS serves critical functions; it facilitates cell-surface interactions, provides protection from pollutants and stressors such as desiccation, and captures nutrients for the biofilm inhabitants (Du et al., 2020). Mature P. aeruginosa biofilms consist of mushroom-shaped bacterial microcolonies surrounded by an extracellular polysaccharide matrix (Mattick, 2002). The overproduction of these substances appears to be a trait commonly exhibited by members of the genus Hymenobacter, and the Icelandic strains in this study are no exception. The copious amounts of EPS produced by these
strains makes them rather difficult to work with in liquid media, as they tend to clump together in large cell aggregates. This is likely the reason for the decrease in optical density observed for many of the *Hymenobacter* wells in the osmotic stress tolerance experiments: the cells clump together, and eventually fall out of solution. This almost constant overproduction of EPS is one of the reasons why they could be considered good candidates for biofilm formation. Furthermore, it could be a consequence of their functional role in the environment. For instance, EPS production may contribute to the structure and integrity of soil particles and as such help to shape the physical environment of *Hymenobacter* strains isolated from soil (Larsbrink and McKee, 2019). Similarly, the complex three-dimensional and structural quality of many lichens is dependent on EPS ‘gluing’ component parts into place. Spribille et al. (2020) suggest that this structure-defining characteristic of lichens should be treated as a complex biofilm, which is contributed to by lichen symbionts and non-phototrophic lichen-associated bacteria alike. Despite these considerations, EPS biosynthesis is an energy-demanding process: while it serves critical functional purposes in natural environments, in laboratory cultures, the production of EPS does not appear to impact cell viability or growth (Costa, Raaijmakers and Kuramae, 2018). Since it is thought that laboratory cultures do not require EPS for survival under optimal conditions, it could be the case that the *Hymenobacter* strains do this to protect themselves against some unknown inhibiting stress. For example, an exciting recent study showed that *Hymenobacter nivis* strain P3T displays a significant growth enhancement when in the presence of light, while dark conditions increased the production of EPS (Terashima et al., 2018). Clearly, more work is required to understand even seemingly simple concepts such as optimum growth conditions for these strains, as well as further developing the relationship between interspecies interactions, ecological stress responses, and functional roles of the *Hymenobacter* strains in their natural environments.

A useful framework for conceptualising the relationship between ecological interactions and stress responses is the C-S-R framework, developed by Ho et al. (2012). This model has been used to relate observations on the life strategies of bacteria to their behaviour and response to their environment. Here, bacteria are assigned as competitors based on their capacity to capitalise on resource availability and therein outcompete others for nutrients and space; stress-tolerators can withstand and persist under conditions of stress, such as low nutrient availability, non-optimal temperatures, pH and salinity; and ruderals are characterised
by their ability to re-colonize environments facing frequent disturbance events, such as desiccation, freeze-thawing and mechanical disturbances (Ho, Lonardo and Bodelier, 2017). Based on the results of this study, *Hymenobacter* strains H16F320 and LW0504 would not be classified as competitors (C), but would rather be positioned under stress tolerators-ruderals (S-R) in the scheme. Although osmotic shock would be defined as a stressor, causing biomass restriction, rather than a disturbance, causing biomass destruction, it naturally follows disturbance events such as freeze-thawing. More research is required on the tolerance of the hymenobacters to such disturbance events, but with our current knowledge, they appear to fall more closely in line with the stress-tolerators, being able to withstand low temperatures, high levels of radiation, and low nutrient availability. The C-S-R continuum allows for the classification of bacteria on the basis of their whole set of ecological traits (Ho et al., 2017). As such, it provides a framework for discussing their various characteristics in the context of their ecological life strategy. For example, the lack of competitiveness exhibited by the hymenobacters may facilitate their preponderance in niches inhospitable to other more competitive populations of bacteria. *Hymenobacter* sp. are often found to be among the most predominant genera in areas characterised by high levels of UV-radiation: this is clearly not because they outcompete all other species, but because other species simply can’t survive there. However, their ability to induce motility in the presence of other species could point to a competitive strategy. Further work is required to determine whether this induced phenotype benefits the competitive ability of the hymenobacters in mixed cultures, perhaps allowing them to escape, or whether it is a social phenomenon of a different nature. The C-S-R framework allows us to relate microbial community dynamics with ecological traits in a way that emphasises their interdependence. The competition sensing hypothesis, the idea that microbes can sense competition with their stress responses, supports this view. Instead of sensing the actual presence of competitors, bacteria use the mechanisms with which they sense environmental stress, for instance by detecting nutrient limitation and cell damage. Competition, by definition, is harmful, and stress responses therefore represent ideal mechanisms for bacteria to detect the presence of competitors (Lories et al., 2020). If this is the case, then community dynamics and environmental stress responses are inextricably linked. Although this study did not provide any evidence for or against the competition sensing
hypothesis, the hypothesis itself does provide convincing arguments for why abiotic and biotic stressors are worth investigating in tandem.
6 Conclusion

Members of the genus *Hymenobacter* are commonly isolated from harsh environments, and are often resistant to abiotic stressors such as cold temperatures and UV radiation. Their extremophilic properties make them attractive for biotechnological purposes: their production of novel pigments, extracellular polymeric substances and enzymes represent reservoirs of vast industrial potential. However, little is known about their response to other environmental stressors, or their functional role in the complex microbial communities from which they are isolated.

Tolerance to osmotic stress has been most extensively studied in *E. coli* and *B. subtilis* as respective representatives of gram-negative and positive bacteria. Gram-negative species usually respond to osmotic stress using the alternative sigma factor RpoS; however, members of the phylum *Bacteroidetes* do not appear to possess this sigma factor, and therefore the mechanisms of their stress tolerance are still largely unknown. It was predicted that the Icelandic *Hymenobacter* strains from this study would be to some extent tolerant of osmotic stress, owing to the environments from which they were isolated: interestingly, they proved to exhibit a total lack of tolerance. Thus, the first research question was definitively answered: the hymenobacters are not able to tolerate osmotic stress, no matter which humectant was used. Further work is required to increase our understanding of the reasons behind this, as well as the responses of the Icelandic *Hymenobacter* isolates to various other environmental stressors. These findings could be of biotechnological significance.

Finally, this study began to provide observations on the nature of the interspecific interactions exhibited by these strains and other co-isolated strains. The second research question asked how the *Hymenobacter* strains perform in competition with co-isolated strains: although this can not be conclusively answered, the results in the present study do point to their lack of competitiveness, placing closer to the stress-tolerator-ruderal (S-R) position in the three-dimensional C-S-R (competition-stress-ruderal) framework. Observations on the negative effects of competition on the growth performance of the *Hymenobacter* strains will be
complemented by culture-independent studies, giving a better idea of the larger community context. Even in the simple assays performed, complex dynamics appeared to emerge, such as the conditional induction of twitching motility. Although this induced phenotype could be a competitive strategy, further work is certainly required to reveal the true nature of and reasons behind the behaviour. Nevertheless, the third research question was concerned with interspecific interactions; clearly, the observation of twitching motility shows that co-culturing the *Hymenobacter* strains with co-isolated strains did, indeed, result in interesting and previously undiscovered characteristics. Relating the interspecific interactions of the hymenobacters to their functional roles within bacterial assemblages is important from an ecological perspective. Studies of this kind will allow us to better understand the complexity of, and eventually even begin to manipulate, natural bacterial communities.
References


Appendix

1. Statistical significance for the osmotic stress growth experiments

Figure 14: Visualisation of t-tests performed for every single timepoint on every growth curve from Fig. 2 against the standard condition curve for the respective strain. In this figure, H16F320 NaCl corresponds to Fig. 2A; Sucrose corresponds to Fig. 2B, etc. Under each heading are four lines; these correspond to the growth curves obtained at the four different water activity levels with each humectant. For instance, in H16F320 NaCl, the first line corresponds to T-tests performed for every timepoint of the NaCl $a_w$ 0.975 curve against the standard conditions $a_w$ 0.989 curve for H16F320. The line below is $a_w$ 0.967; the next line is 0.955; and finally, 0.946. The black lines at the bottom represent timepoints on the growth curves; the shorter lines represent 25 hours, while the longer lines demarcate every 50 hours of the experiment. The colours represent the results of the t-tests, with green indicating statistical significance ($p < 0.05$).

Figure 14 shows statistical significance of the results from Fig. 2, plotted against the time course of the growth experiment. Statistical significance is reported in the form of t-tests with p-values < 0.05, shown in green. Fig. 14 determines whether the difference in optical density between the standard curve and all other curves is statistically significant, and whether this changes over time. For instance, the first line, corresponding to the curve obtained via growth at $a_w$ 0.975 in Fig. 2A, shows that after the first 10 hours of the experiment, the curve becomes
significantly different from the standard conditions curve and stays that way for the duration of the experiment. For JF1031, statistical significance is not reached until nearly the 50-hour mark in most cases; this reflects the long lag time of JF1031, which did not start growing under standard conditions until after this point. For LW0504, statistical significance is reached after 25 hours, and every curve is statistically significant from the standard conditions curve, apart from one. This curve corresponds to $a_w 0.955$ in Fig. 2; as mentioned earlier, this curve is most likely the result of contamination of one of the triplicate samples. Using this measure of statistical significance over time, erroneous results can be confidently discounted. Moreover, while Fig. 2 suggests that strains H16F320, JF1031 and LW0504 are not resistant to osmotic stress, Fig. 14 confirms that the results are meaningful.

Figure 15: Visualisation of t-tests performed for every single timepoint on every growth curve from Fig. 3 against the standard condition curve for the respective strain. In this figure, MAS034 NaCl corresponds to Fig. 3A; Sucrose corresponds to Fig. 3B, etc. Under each heading are four lines; these correspond to the growth curves obtained at the four different water activity levels with each humectant. For instance, in MAS034 NaCl, the first line corresponds to t-tests performed for every timepoint of the NaCl $a_w 0.975$ curve against the standard conditions $0.989$ curve for MAS034. The line below is $a_w 0.967$; the next line is 0.955; and finally, 0.946. The black lines at the bottom represent timepoints on the growth curves; the shorter lines represent 25 hours, while the longer lines demarcate every 50 hours of the experiment. The colours represent the results of the t-tests, with green indicating statistical significance ($p < 0.05$).

Figure 15 reveals that in almost all cases, the curves obtained from Fig. 3 in all media conditions with lowered water activity are not significantly different from the curves obtained under standard conditions. Statistical significance was reached after the 75-hour mark in Fig. 3A $a_w 0.955$, corresponding to MAS034 and NaCl; this curve is most likely the result of contamination. Similarly, the 0.967 curve from Fig. 3D, corresponding to MAS040 and NaCl, becomes significantly different from the standard conditions curve after the 100-hour mark. This curve is completely flat, but unlike the others starts at a higher OD, possibly due to a
greater inoculated cell count. Overall, while the growth curves in Fig. 3 do suggest that the strains are not osmotolerant, more replicates for MAS034 and MAS040 under all conditions are required in order to assert this with confidence. Fig. 15 shows that the differences in growth for MAS040 between standard conditions and lowered water activity were not statistically significant. However, t-tests revealed that the differences in AUC under standard conditions between MAS040 and H16F320, JF1031 and LW0504 were statistically significant (p = 0.0019; 0.0012; 0.0097). This was not the case for MAS034 (p > 0.05).

![Figure 16: Visualisation of t-tests performed for every single timepoint on every growth curve from Fig. 4 against the standard condition curve for the respective strain. In this figure, H16F320 NaCl corresponds to Fig. 4A; Sucrose corresponds to Fig. 4B, etc. Under each heading are four lines; these correspond to the growth curves obtained at the four different water activity levels with each humectant. For instance, in H16F320 NaCl, the first line corresponds to T-tests performed for every timepoint of the NaCl a_w 0.987 curve against the standard conditions 1.003 curve for H16F320. The line below is a_w 0.968; the next line is 0.960; and finally, 0.950. The black lines at the bottom represent timepoints on the growth curves; the shorter lines represent 25 hours, while the longer lines demarcate every 50 hours of the experiment. The colours represent the results of the t-tests, with green indicating statistical significance (p < 0.05).](image)

Looking at statistical significance over time in Figure 16, all curves from Fig. 4 follow largely similar trajectories when compared to the standard conditions curve of their respective strains. Strain H16F320 achieves significant differences in growth after around 75 hours in most cases. At this point, the growth exhibited under standard conditions becomes statistically different to the rest of the curves, which display very little to no growth. This is due to variation in the replicate growth curves under standard conditions, meaning that a certain OD threshold had to be reached before the growth could be considered meaningfully different to the curves.
showing no growth. In comparison, differences in growth for strain JF1031 become significant around the 50 hour mark, despite the fact that the bacterium has only slightly started to grow under standard conditions at this point. This is due to the fact that the replicates have very similar growth curves, and so significance can be more easily attributed to this strain. Importantly, the second glycerol ($a_w$ 0.975) line for strain JF1031 (corresponding to Fig. 4F) exhibits statistical significance between 50 to 100 hours; the 50-hour mark is when JF1031 begins to grow under standard conditions. At the 100-hour mark, JF1031 begins to grow in the glycerol $a_w$ 0.975 media, and at this point, the difference between the two curves starts to lose statistical significance. This is not because of variation in the replicates, but because the two curves are too similar to be considered different statistically; this contributes to the notion that strain JF1031 was able to grow under this condition. The difference in AUC between the two curves is statistically significant ($p = 0.048$), and so this can be considered to be a valid result.

Figure 17: Visualisation of t-tests performed for every single timepoint on every growth curve from Fig. 5 against the standard condition curve for the respective strain. In this figure, AR0120 NaCl corresponds to Fig. 5A; Sucrose corresponds to Fig. 5B, etc. Under each heading are four lines; these correspond to the growth curves obtained at the four different water activity levels with each humectant. For instance, in AR0120 NaCl, the first line corresponds to T-tests performed for every timepoint of the NaCl $a_w$ 0.987 curve against the standard conditions 1.003 curve for AR0315. The line below is $a_w$ 0.968; the next line is 0.960; and finally, 0.950. The black lines at the bottom represent timepoints on the growth curves; the shorter lines represent 25 hours, while the longer lines demarcate every 50 hours of the experiment. The colours represent the results of the t-tests, with green indicating statistical significance ($p < 0.05$).

The statistical significance against time graph shown above in Figure 17 is the same as those presented previously, but requires slightly different interpretation. This is because for all other strains, the standard condition curve represented optimum conditions and all other curves were compared to this; since growth did not occur under most conditions, the statistical significance graphs were used to confirm that curves showing no growth were significantly
different to curves showing growth. In Fig. 5, however, all curves show growth; and indeed, the standard condition curve often represents the worst growth, particularly when NaCl is used as the humectant. So, statistical significance here denotes that curves showing growth at lowered aw are significantly different to the standard curve, showing little growth. If hypothetically, growth was reduced linearly as water activity decreased, it could perhaps be expected that curves at higher aw would be less statistically significant than curves at lower aw, because the rate of growth and carrying capacity would change more as water activity got lower. This is not the case for AR0120, for which the standard curve is not significantly different to any other curve, barring a short duration between 25 and 50 hours for NaCl aw 0.968, represented in Fig. 5A. This is probably due to variation in the replicates; more replicates would be needed to achieve significantly different growth curves. For AR0315, statistical significance was much more readily attained. Interestingly, for NaCl, the curves which were most similar to the standard conditions curve were significantly different from it. For glycerol, all curves were significantly different to the standard conditions curve. Thus, the differences in growth curves for AR0315 can be said to be statistically significant and that water activity has a pronounced effect on growth.
2. Growth parameters from the osmotic stress growth experiments

Table 4: Growth parameters obtained with Growthcurver in RStudio by fitting the logistic equation with the experimental growth curves from the first osmotic stress tolerance investigation. $a_w$ represents water activity; $r$ represents the intrinsic growth rate; $K$ represents the carrying capacity of the cell population.

<table>
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<th>Hum.</th>
<th>$a_w$</th>
<th>r</th>
<th>K</th>
<th>r</th>
<th>K</th>
<th>r</th>
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<th>r</th>
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<td>0.020</td>
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Table 5: Growth parameters obtained with Growthcurver in RStudio by fitting the logistic equation with the experimental growth curves from the second osmotic stress tolerance investigation. $a_w$ represents water activity; $r$ represents the intrinsic growth rate; $K$ represents the carrying capacity of the cell population.

<table>
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<tr>
<th>Hum.</th>
<th>$a_w$</th>
<th>r</th>
<th>K</th>
<th>r</th>
<th>K</th>
<th>r</th>
<th>K</th>
<th>r</th>
<th>K</th>
<th>r</th>
<th>K</th>
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<td>0.193</td>
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<td>0.468</td>
<td>0.062</td>
<td>0.554</td>
<td>0.173</td>
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<td>0.012</td>
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<td>0.007</td>
<td>0.021</td>
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<td>0.004</td>
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<td>0.007</td>
<td>0.001</td>
<td>0.010</td>
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<tr>
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<tr>
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Table 6: AUC (area under the curve) for each experimental growth curve from the first osmotic stress tolerance investigation. Obtained with the Growthcurver package in RStudio.

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<th>H16F320</th>
<th>JF1031</th>
<th>LW0504</th>
<th>MAS034</th>
<th>MAS040</th>
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<td>3</td>
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<td>0.961</td>
<td>0.978</td>
</tr>
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<td>0.966</td>
<td>0.954</td>
</tr>
<tr>
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<td>0.966</td>
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<tr>
<td>Glycerol</td>
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<td>0.963</td>
<td>0.955</td>
<td>0.966</td>
<td>0.954</td>
</tr>
</tbody>
</table>

Table 7: AUC (area under the curve) for each experimental growth curve from the second osmotic stress tolerance investigation. Obtained with the Growthcurver package in RStudio. The data for glycerol a_0, 0.978 is omitted as the stock solution itself, and therefore all resulting cultures, was contaminated.

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<th>LW0504</th>
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<td>0.961</td>
<td>0.961</td>
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<tr>
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<td>0.961</td>
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<td>0.987</td>
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<tr>
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<td>0.961</td>
<td>0.961</td>
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<td>0.987</td>
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<tr>
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<td>0.961</td>
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