THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Exploration and discovery of enzymes and non-catalytic proteins targeting microbial α-glucans

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Cover:
AlphaFold structure of *Fj*CBMXXX$_{\mathrm{GH87}}$, a carbohydrate-binding module studied in this thesis, see chapter 4.1.4 and Article III.

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Preface

This dissertation serves as partial fulfilment of the requirements for obtaining the degree of Doctor of Philosophy at the Department of Life Sciences at Chalmers University of Technology. The work was primarily funded by the Swedish Research Council (Vetenskapsrådet: project 2020-03618). The PhD studies were carried out between July 2021 and February 2026 under the supervision of Professor Johan Larsbrink and co-supervision of Dr. Scott Mazurkewich and Associate Professor Lauren McKee. The thesis was examined by Associate Professor Elin Esbjörner Winters.

The majority of the work in this thesis was carried out at the Division of Industrial Biotechnology (IndBio) at Chalmers University of Technology. Isothermal titration calorimetry experiments were performed at Chemistry and Chemical Engineering at Chalmers University of Technology, and at the University of Michigan by Lake Shimer and Nicole Koropatkin. Crystallization experiments were performed at Gothenburg University and X-ray diffraction data were collected at the MAX IV Laboratory by Dr. Scott Mazurkewich.

Abstract

Polysaccharides are everywhere. Many have structural or energy-storage roles, such as the well-known plant polysaccharides cellulose and starch. This thesis focuses on the less studied α-1,3-glucans, which can be found in fungal cell walls and bacterial biofilms fulfilling various functional roles. In fungal cell walls, α-1,3-glucans are known to function as a storage polysaccharide in some species, and as virulence factor in others. In dental plaque, α-1,3-glucans build up the protective structure and provide adhesion to the tooth, contributing to the characteristic stickiness and resilience of biofilms. Because of their structural importance and involvement in pathogenicity, there is interest in the degradation of α-1,3-glucan. The aim of my thesis studies was to increase the knowledge concerning which enzymes can degrade α-1,3-glucan (mutan) and mixed-linkage α-1,3/α-1,6-glucan (alternan) and how these enzymes function.

I have explored the two carbohydrate-active enzyme families currently known to contain α-1,3-glucanase activity: glycoside hydrolase families (GH) 71 and 87. Two GH71 enzymes from the fungus *Aspergillus nidulans* were studied. Through structural determination coupled with biochemical characterization we elucidated the mechanisms and catalytic amino acids for these enzymes. I also studied a GH87 enzyme from *Flavobacterium johnsoniae* and discovered that one of its appended domains is a novel carbohydrate-binding module. By binding both mutan and alternan, its presence significantly improves the activity of the enzyme compared to the lone catalytic domain.

I also discovered new sources of mutan- and alternan-degrading enzymes via screening of environmental soil samples to identify microorganisms able to grow on these polymers. Six *Streptomyces* isolates were chosen for further study, which led to the description of two new species, *Streptomyces castrisilvae* and *Streptomyces glycanivorans.* In addition, analysis of the secretome of two of the isolates during growth on mutan and alternan showed that both strains employed several GH87 enzymes, all with different domain architecture, for the degradation of both polysaccharides. Growth on alternan also induced the expression of a gene cluster whose proteins' concerted actions were shown to facilitate alternan and dextran (α-1,6-glucan) utilization.

Overall, my work contributes to the understanding of carbohydrate-active enzymes and carbohydrate-binding modules targeting microbial α-1,3-glucans.

List of publications

I. Mazurkewich, S., **Widén, T**., Karlsson, H., Evenäs, L., Ramamohan, P., Wohlert, J., Brändén, G., and Larsbrink, J. (2025) Structural and biochemical basis for activity of *Aspergillus nidulans* α-1,3-glucanases from glycoside hydrolase family 71. *Commun. Biol.* **8**, 1298. doi: 10.1038/s42003-025-08696-3

II. **Widén T**, McKee L, Koropatkin N, Larsbrink J. Characterization of an α-glucan-binding module from *Flavobacterium johnsoniae* as a founding member of carbohydrate-binding module family XXX. Manuscript.

III. **Widén, T.**, Rangel, A. T., Lombard, V., Drula, E., Mazurkewich, S., Terrapon, N., Kerkhoven, E. J., and Larsbrink, J. (2024) *Streptomyces castrisilvae* sp. nov. and *Streptomyces glycanivorans* sp. nov., novel soil streptomycetes metabolizing mutan and alternan. *Int. J. Syst. Evol. Microbiol.* **74**(9):006514. doi: 10.1099/ijsem.0.006514

IV. **Widén T**, Forsberg Z, Shimer L, Eijsink V, Koropatkin N, Larsbrink J. Comparative proteomics and enzymatic analyses of soil *Streptomyces* growing on mutan and alternan reveals a gene cluster involved in alpha-1,6-glucan degradation. Manuscript.

Contribution summary

I. Second author. I conducted large parts of the biochemical experimental work together with my co-authors, participated in the data analysis and wrote parts of the manuscript.

II. First author. I took part in the project conceptualisation, planned and performed the experimental work. I analysed the data, interpreted the results and wrote the manuscript together with my co-authors.

III. First author. I took part in the project conceptualisation, planned and performed the majority of the experiments, analysed the data, interpreted the results and wrote the manuscript together with my co-authors.

IV. First author. I took part in the project conceptualisation, planned and performed the majority of the experiments, analysed the data, interpreted the results and wrote the manuscript together with my co-authors.

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Abbreviations

AA	Auxiliary activities
ABC-transporter	ATP-binding cassette transporter
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
CAZy	Carbohydrate-active enzymes database
Cazyme	Carbohydrate-active enzyme
CBM	Carbohydrate-binding module
ESBP	Extracellular solute-binding protein
GH	Glycoside hydrolase
gpPUL	Gram-positive polysaccharide utilization locus
Ig	Immunoglobulin
LPMO	Lytic polysaccharide monooxygenase
ORF	Open reading frame
PDB	Protein Data Bank
PUL	Polysaccharide utilization locus
ROK-family protein	Repressor, ORF, kinase family protein

Chapter 1: Introduction

Carbohydrates represent one of the most structurally diverse classes of biomolecules, owing to the numerous ways in which monosaccharides can be assembled into di-, oligo-, and polysaccharides (1). Fulfilling important roles in all living organisms, carbohydrates can function as storage molecules, such as starch in plants and glycogen in animals, act as key structural components, such as cellulose in plants and chitin in fungi and insects, and also mediate processes such as cell-to-cell recognition and signalling (2). While many abundant polysaccharides have been extensively studied, others have received much less attention despite their biological significance. One such group is the α-1,3-glucans, polysaccharides composed of glucose units linked by α-1,3-glycosidic bonds, which can be found in fungal cell walls and dental plaque biofilms where they contribute to structural integrity, energy storage, and, in some cases, pathogenicity (3, 4).

The synthesis, modification and degradation of polysaccharides are carried out by carbohydrate-active enzymes (cazymes). Reflecting the complexity of carbohydrates, there is a vast diversity within this group of enzymes, that act on different kinds, parts and bonds of carbohydrates. However, similar to studies on α-1,3-glucans themselves, enzymes targeting them remain poorly understood with only a few representatives biochemically characterized.

1.1 Aim of this thesis

The overall aim of this thesis was to expand current knowledge of enzymes and proteins that target microbial α-1,3-glucans. To address this overarching goal, two complementary research strategies were employed: a targeted investigation building upon existing knowledge of α-1,3-glucan-active enzymes, and an exploratory, activity-driven approach to identify previously uncharacterized enzymes exhibiting α-1,3-glucanase activity.

For the first strategy, the two glycoside hydrolase families (GH) known to contain α-1,3-glucanase activity, **GH71 and GH87**, were explored. Both families were at the time of study underexplored, with only a few characterized members, and there was no structural information for any enzymes from GH71. In addition to catalytic domains, many carbohydrate-active enzymes contain **carbohydrate-binding modules** (CBMs) targeting the same substrate and an additional objective was therefore to study the occurrence and functionality of putative binding modules.

Article I focuses on enzymes from GH71, providing the first crystal structure with ligands in the family. This result, coupled to biochemical characterisation, confirms the catalytic mechanism and conclusively identifies the catalytic amino acid residues of these types of enzymes.

Article II describes the characterisation of a GH87 enzyme from the Gram-negative bacterium *Flavobacterium johnsoniae*, and especially focuses on a novel carbohydrate-binding module within this protein which was shown to have affinity for α-1,3-glucan. This domain belongs to a previously unknown CBM family, and its characterisation provides a foundation for the studies of other proteins in the same family as well as a functional characterization of specific module studied.

For the second, exploratory approach, I searched for α-1,3-glucanase activity in nature. **Article III** focused on the isolation, screening and characterization of α-1,3-glucan-degrading bacteria, to provide insight into the carbohydrate metabolism of selected species.

In **Article IV**, proteomics was employed to study two of the isolated organisms' protein production during their growth on α-1,3-glucan and alternan (mixed-linkage α-1,3/α-1,6-glucan). Several proteins involved in carbohydrate degradation were identified, including proteins encoded by a gene cluster that was only present in the samples from cultivations on alternan. The proteins of this cluster were studied in detail, establishing the cluster as a Gram-positive polysaccharide utilization locus associated with alternan degradation.

In this thesis, I first provide a background concerning microbial α-1,3-glucans (chapter 2) and carbohydrate-active enzymes (chapter 3). I then present my research findings together with relevant background of the field (chapter 4) and discuss future outlooks and applications in the field (chapter 5).

Chapter 2: Microbial polysaccharides

Carbohydrates are of fundamental importance to microbial life. They serve as energy sources that can be acquired from external substrates (e.g. starch) through targeted degradation and uptake, as well as intracellular storage compounds (e.g. microbial glycogen) that support survival under fluctuating environmental conditions. In addition, carbohydrates constitute essential structural components for microorganisms, both as polysaccharides building up the cell wall, but also, for some organisms, as extracellular polymers contributing to the adhesive and protective properties of biofilms. A type of polysaccharide that can play all these roles, in different contexts, is α-1,3-glucan, which is principally known to be present in the biofilm dental plaque and fungal cell walls. In the following sections, I describe these structures and the known roles of α-1,3-glucan and related polysaccharides in these.

2.1 The biofilm dental plaque

Dental plaque in oral diseases

Oral diseases are estimated to affect 3.5 billion people in the world, with 90 % of the world's adult population being affected by gingivitis (reversible gum inflammation), 50 % by periodontitis (severe gum inflammation that can lead to tooth loss), and over a third of the world's population is living with untreated dental caries (tooth decay) (5–7). The fundamental cause of many oral diseases, including caries, gingivitis, and periodontitis, is untreated dental plaque (5, 6). Dental plaque is a biofilm infection and is, like most biofilms, difficult and costly to treat because of its resilient nature. The teeth are non-shedding surfaces, i.e. there is no removal of their outer surface and thereby the biofilm with time. The biofilm consists of bacteria, exopolysaccharides, extracellular DNA, and proteins, creating a sticky structure that is difficult to remove (8). Small amounts of dental plaque may not be detrimental to oral health, but if the biofilm accumulates it causes irritation and inflammation, i.e. gingivitis (6). Gingivitis is the first stage of periodontitis, and while not everyone affected by gingivitis may develop periodontitis, periodontitis is always preceded by gingivitis. The development of periodontitis is dependent on several risk factors, such as genetic predisposition, nutrition, stress, and, most importantly, dental plaque (5). The severity of gingivitis is directly correlated with the amount and maturity of the dental plaque, making plaque removal the strategy for reduction of gingivitis and prevention of periodontitis (5).

While periodontitis is mainly caused by dental plaque on and under the gingival margin, dental plaque both under and over the gingival margin (sub- and supragingival plaque) can lead to caries (9). In the case of caries, bacteria such as *Streptococci* and *Lactobacilli* ferment sugars for the production of ATP with lactic acid as a byproduct. This leads to localized acidic microenvironments within the plaque that ultimately demineralize the enamel of the teeth (4, 9).

Structure of dental plaque

The biofilm structure is essential for many functions in dental plaque. It promotes adhesion to the teeth, enables signalling and nutrient sharing between the organisms in the microbial community, and blocks diffusion of buffering saliva creating protected microenvironments (10). The biofilm consists of various microorganisms embedded in the extracellular matrix consisting of proteins, DNA, and polysaccharides, all of which fulfil important roles in the plaque (Fig 2.1).

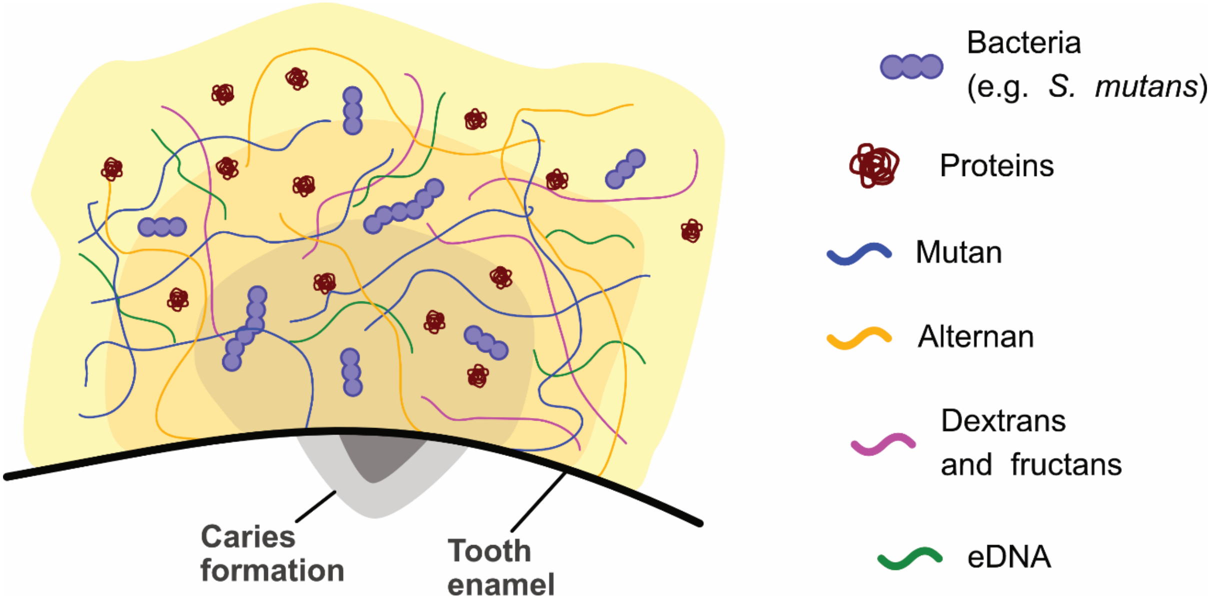


Figure 2.1. Schematic illustration of dental plaque. The biofilm consists of a complex mixture of bacteria, proteins, extracellular DNA and polysaccharides, together creating an adhesive and protective structure.

Dental plaque is not an infection in the sense that a foreign pathogen invades a tissue; rather, the biofilm formation is caused by a change in the microbial community composition (9). The healthy oral cavity contains a multitude of commensal bacteria and fungi, which are important to oral health in several ways. Some commensal bacteria act directly antagonistic against pathogens, by producing antimicrobial compounds, such as hydrogen peroxide that can counter the lowering of the pH associated with acidogenic bacteria, and additionally, simply the presence of commensal bacteria inhibits the colonization by pathogens by reducing available binding surfaces (6, 11, 12). In the transition between a healthy oral microbiome and dental plaque, the microbial community goes from being diverse to being dominated by acidogenic (acid-producing) and aciduric (acidic-tolerating or preferring), and often anaerobic bacteria. Critical to this shift is the intake of dietary sugars, which leads both to the synthesis of exopolysaccharides, which provides a structure for bacteria to colonize and the establishment of anaerobic microenvironments, and to fermentation, which causes acidification and further selection of aciduric bacteria. Especially correlated with caries is *Streptococcus mutans*, other *Streptococci* and *Lactobacilli* (9).

In the extracellular matrix, extracellular DNA is believed to be important for the initial formation of the plaque, having a role in adhesion between bacteria and the enamel, but its exact role is not elucidated (13). Proteins in the extracellular matrix fulfil various roles, including providing adhesion between cells, enamel and polysaccharides, and the acquisition of nutrients and matrix turnover by degradative enzymes (8, 14).

The perhaps most characteristic proteins of dental plaque, however, are the transglycosylases that synthesize the extracellular polysaccharides that make up around 10-20 % of the dental plaque dry weight (15). The transglycosylases, often referred to as glucosyltransferases (GTFs) and fructosyltransferases (FTFs), produce glucans and fructans from sucrose. It should be noted that these enzymes are in fact not bona fide glycosyltransferases which utilize sugar phosphates as glycosyl donors, but instead glycoside hydrolases with transglycosylase activity. Fructans are soluble and mostly contribute to virulence of the plaque by being a nutrient source for the bacteria, though it may also contribute to adhesion between bacteria and the polysaccharides (16). In contrast, the glucans can be both soluble and insoluble depending on the linkages and branching of the polysaccharide. The variations of the polysaccharides are a result of varying GTFs and environmental conditions. *S. mutans* alone produces at least three different GTFs (GtfB, GtfC, GtfD) which form distinct polysaccharides, and the related species *Streptococcus salivarius* at least four (GtfJ, GtfK, GtfL, GtfM) (15, 17–19). The solubility of the resulting polysaccharides seems to be mostly dependent on the proportion of α-1,3- and α-1,6-linkages, where α-1,6-linkages are associated with higher solubility. The composition of the polysaccharides is further complicated by the fact that some GTFs prefer to append the polysaccharide being synthesized to other oligo- or polysaccharides, such as starch hydrolysates, dextran, or fructan, which leads to a complex mixture of linkages and monosaccharide building blocks in the resulting matrix. Because of this, the nomenclature regarding these polysaccharides sometimes gets confusing, and they are sometimes broadly referred to as mutans, dextrans or α-glucans (18, 20, 21). In my work I refer to the mostly linear α-1,3-linked polysaccharide, produced by the GtfB enzyme from *S. mutans* and GtfJ enzyme from *S. salivarius* as α-1,3-glucan or mutan (Fig. 2.2 A). The polysaccharide consisting of alternating α-1,3-/α-1,6-linked glucose units, such as the one produced from the GtfL enzyme from *S. salivarius*, I refer to as alternan (Fig. 2.2 B). Lastly, I refer to the α-1,6-linked glucan produced by GtfD from *S. mutans* as dextran.

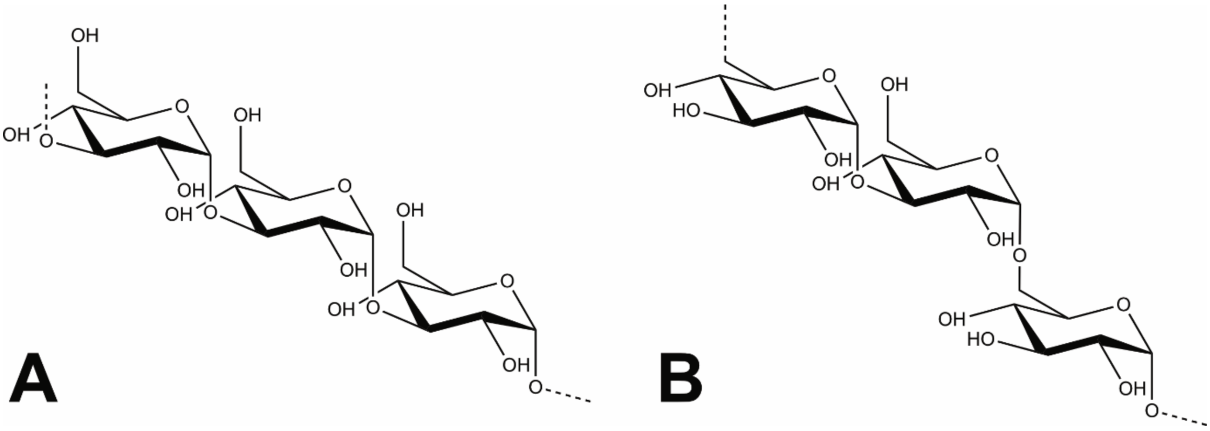


Figure 2.2 Chemical structure of **(A)** α-1,3-glucan and **(B)** alternan (mixed-linkage α-1,3/1,6-glucan).

In dental plaque, soluble polysaccharides such as dextran have been shown to have more of a storage molecule role (15), while the insoluble polysaccharides are considered more structurally important for the establishment and growth of the biofilm (22). Degradation of these insoluble polysaccharides is therefore a promising strategy for disruption or dispersion of the biofilm (23).

2.2 Fungal cell walls

Another biological structure of high importance that includes α-1,3-glucans are fungal cell walls. Fungal cell walls are highly complex structures, essential for the viability and growth of fungi (24, 25). The walls have some similarity to plant cell walls, and they consist mainly of polysaccharides and proteins that attach to the cell membrane through connections to intermembrane proteins. The cell wall needs to provide both strength and elasticity, protecting the cell from outer threats and outward directed turgor pressure but at the same time allowing for sensing and uptake of nutrients. It is the cell wall that defines the shapes of fungi, giving rise to the multitude of micro- and macroscopic shapes they can take, such as hyphae, conidia, spores, unicellar yeasts and multicellular fruiting bodies. The composition of the fungal cell wall varies between species and is highly dynamic, generally employing over a thousand of genes for its assembly and regulation (25). The composition may change in response to environmental factors such as humidity, pressure, nutrient access and other perturbations (24). In *Aspegillus fumigatus*, this adaptability was showcased in a study showing that mutant strains, devoid of either α-1,3-glucan, chitin, galactomannan or galactosaminogalactan, compensated for the lack of one polysaccharide through the production of another, though the morphology was affected to various degrees (26).

Despite the high variability of fungal cell walls, some foundational characteristics are more or less conserved and are exemplified in Fig. 2.3 by a depiction of the cell wall of *A. fumigatus.* Most fungi have an inner region of the cell wall whose framework is composed of chitin (β-1,4-linked *N*-actylglucosamine) and β-1,3-glucan. Chitin forms antiparallel chains making up rigid fibrils that provides a strong but inelastic foundation. The chitin fibrils are covalently cross-linked together with β-1,3-glucan triple helices, that include β-1,6-branches which introduce more flexibility (24, 25). Together, chitin and β-glucan create a scaffold around the cell to which proteins and other polysaccharides may bind (24). The outer part of cell wall is more variable, both between species and during adaptations to the environment (24, 27). It can provide properties such as hydrophobicity and adhesion (24). The outer part of the cell wall is connected to the inner part through bonds between the β-glucan and the decorating polysaccharides, (e.g. mannan, galactosaminoglycan, α-1,3-glucan) or through proteins embedded in inner cell wall that are heavily glycosylated, and the glycosylation, usually mannan or galactomannan, making up the outer layer (25).

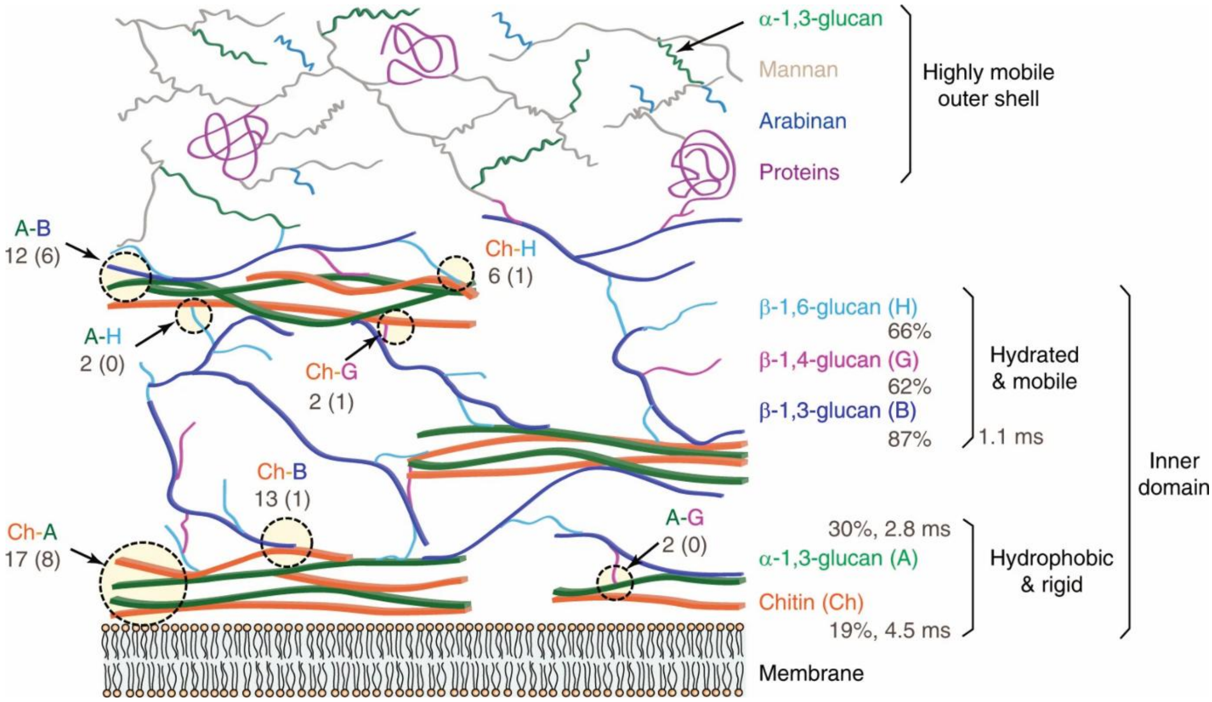


Figure 2.3. Fungal cell wall composition of *A. fumigatus*. The inner region of the cell wall consists of chitin, β-glucan and α-1,3-glucan, while the outer region is more mobile, containing additional decorating polysaccharides and proteins. Figure from (28) under the CC BY 4.0 licence.

α-1,3-glucan in fungal cell walls

Although not present in all fungi, α-1,3-glucan polysaccharides fulfil various important roles across both pathogenic and non-pathogenic fungi (3). In several *Aspergilli* species, α-1,3-glucan has been shown to function as a hyphal aggregation factor, where cells grow dispersed instead of as entangled hyphal pellets in α-1,3-glucan-deficient strains (3). An example of how the cell wall composition may change due to nutrient availability is the presence of α-1,3-glucan in *Aspergillus nidulans*. It has been shown that the polysaccharide functions as a storage molecule, that accumulates during growth and can be consumed again during carbon starvation (29). In the pathogen *A. fumigatus*, α-1,3-glucan is present throughout the cell wall, seemingly an important, but not essential, part of the structure (26). It also contributes to *A. fumigatus*'s virulence, although the exact mechanisms are not entirely elucidated, it has been shown that strains without α-1,3-glucan are less virulent compared to the wildtype (30). Similarly, links between the presence of α-1,3-glucan and virulence can be drawn in species such as *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis,* but the reasons are unclear (31, 32).

In some pathogens, α-1,3-glucan has an indirect role in virulence. In *Histoplasma capsulatum* and the plant pathogen *Magnaporthe grisea,* α-1,3-glucan masks the cell wall β-1,3-glucan from being recognized by the host immune system (33, 34). Another example is *Cryptococcus neoformans* which can cause severe infections, primarily in immunocompromised individuals, usually starting in the lungs but can spread to cause fatal meningoencephalitis (brain tissue inflammation) (35). Key to *C. neoformans*'s virulence and survival within the host is its polysaccharide capsule, consisting of glucuronoxylomannan. This capsule is attached to the cell wall α-1,3-glucan, and if the gene synthesizing α-1,3-glucan is disrupted, the cells can no longer maintain the capsule even though they continue to produce the capsule components (36).

Consequently, it is clear that α-1,3-glucan represent an integral part in the cell walls of certain fungi, with importance both for physiological functions and for host interactions of pathogens.

Chapter 3: Carbohydrate-active enzymes

In the same way as carbohydrates are found everywhere, so are enzymes that break them down, build them up, or modify them. Because of the complexity and variations in carbohydrates, with a multitude of monosaccharide types that can be combined and linked in various ways (37, 38), the number of enzymes targeting them is vast (1, 39, 40). Carbohydrate-active enzymes (cazymes) are present and fulfil important roles in animals (including humans); for example, there are digestive enzymes (e.g. amylases, lactases), enzymes involved in the synthesis and degradation of the energy storage molecule glycogen, and enzymes responsible for glycosylation of proteins. Animals however encode a limited set of degradative cazymes compared to microorganisms, which are regarded as the main decomposers of biomass in nature, whether it is from plants, animals, fungi or other microbes (41). Because of the vast diversity of microorganisms existing in different environments, a multitude of strategies for carbohydrate utilization has evolved (42, 43). An extensive repertoire of enzymes can for instance be found in our gut microbiome, where bacteria symbiotically degrade carbohydrates that would otherwise be indigestible by us alone i.e. dietary fibre (44).

There are many ways of classifying enzymes and probably the most generally applicable is based on their activity, as is done in the Enzyme Commission (EC) number classification system (45). While the EC system is broadly useful it does not encompass all information needed for deeper analysis of enzymes. For example, enzymes may act on multiple substrates, which many cazymes do, and this complicates assignment of their “correct” designation. Since the enzymes are sorted by activity there are many cases where enzymes with very different sequences and structures catalyse the same reaction but with different mechanisms, and these are still categorized together (46, 47). To gather information specifically about cazymes, the carbohydrate-active enzymes database (CAZy, www.cazy.org) was created in 1998, and is kept up to date through active curation and regular updates (48, 39).

3.1 The CAZy classification

In CAZy, cazymes are classified and organized based on sequence similarity. The enzymes on CAZy can be involved in the synthesis, modification, and breakdown of complex carbohydrates and are grouped into five major classes:

- Glycoside hydrolases: Catalyse hydrolytic cleavage of glycosidic bonds. There are currently 194 GH families described, some of which contain multiple subfamilies.
- Glycosyltransferases: Catalyse the formation of glycosidic linkages utilizing sugar phosphates as glycosyl donors. There are currently 139 glycosyltransferase families described.
- Polysaccharide lyases: Catalyse cleavage of uronic acid-containing polysaccharides through a β-elimination mechanism. There are currently 44 polysaccharide lyase families described, some of which contain subfamilies.
- Carbohydrate esterases: Catalyse deacylation of ester-substituted saccharides. There are currently 21 carbohydrate esterase families described.
- Auxiliary Activities (AAs): The AA class contains both enzymes that act directly on carbohydrates and those that act indirectly, i.e. have activities that assist in the catalytic activities of other cazymes, for example by degrading lignin- or lignin-derived compounds. Currently this class contain 18 families, including lytic polysaccharide monooxygenases (LPMOs) and ligninolytic enzymes (49).

An advantage of sequence-based classification is that it is possible to classify proteins that have not yet been biochemically characterized. In a sequence-related family of proteins it also follows that the structures are conserved, providing a basis for structural determination and analysis (47). While many CAZy families are polyspecific, i.e. contain enzymes with varying substrate specificity, the mechanism and catalytic residues are (with few exceptions) conserved and thus predictable (1, 47). Similar families of GHs are also grouped into clans which have similar folds and catalytic amino acids, and therefore operate by the same mechanisms (1). Despite the significant advances in sequencing technologies and the resulting explosion in the amount of available data, CAZy and its classification and documentation of cazymes remain the foundation of the field, owing to the strength of its predictive framework, biological relevance, and rigorous curation and updating (1, 39).

The enzymes studied in this thesis are almost exclusively GH enzymes, and therefore, this class is described in further detail below.

3.2 Glycoside hydrolases

GH enzymes catalyse the hydrolysis of the glycosidic linkage in glycosides, such as those found in oligo- and polysaccharides. GH enzymes can be either endo- or exo-acting, i.e. cleave the substrate at an internal or terminal linkage of the saccharide chain, respectively (Fig. 3.1 A). Which mode of action an enzyme has is largely dependent on its active site topology. An endo-acting enzyme needs to have an open groove or cleft allowing for the (semi-)random binding of the internal part of a saccharide chain (50). Conversely, exo-acting enzymes that specifically target the polymer end often have pocket-like active sites. In many cazymes, one or more substrate-binding sites can be identified, and these are commonly referred to as subsites. These are numbered according to their position relative to the glycosidic bond that is cleaved. Subsites are designated with negative numbers toward the non-reducing end of the substrate and positive numbers toward the reducing end, with bond cleavage occurring between the −1 and +1 subsites (51) (Fig. 3.1 B).

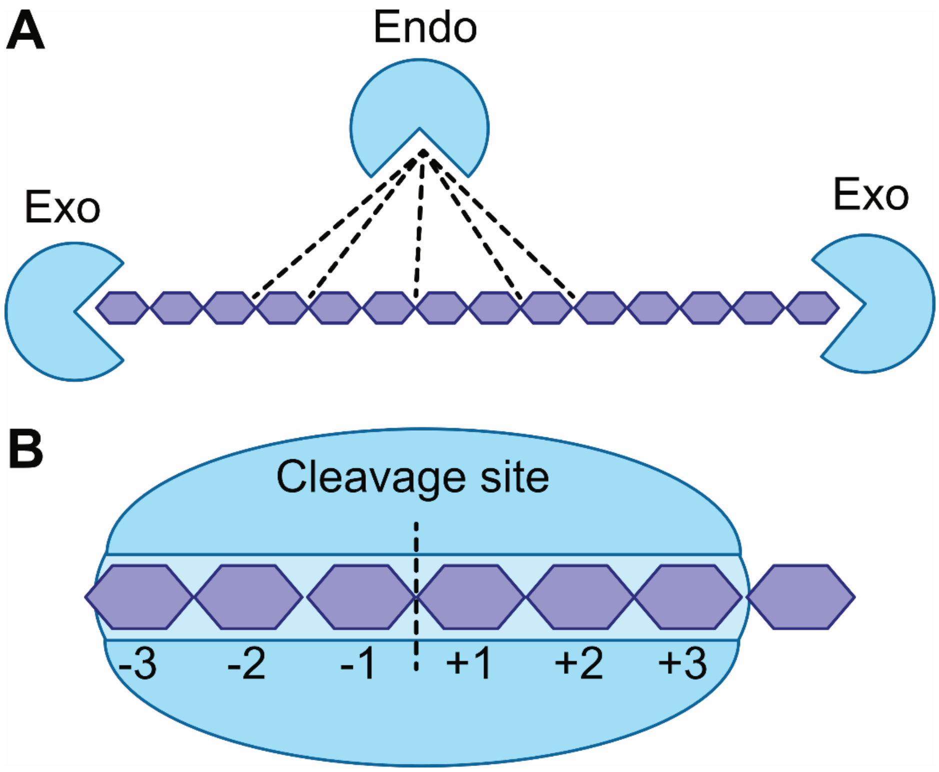
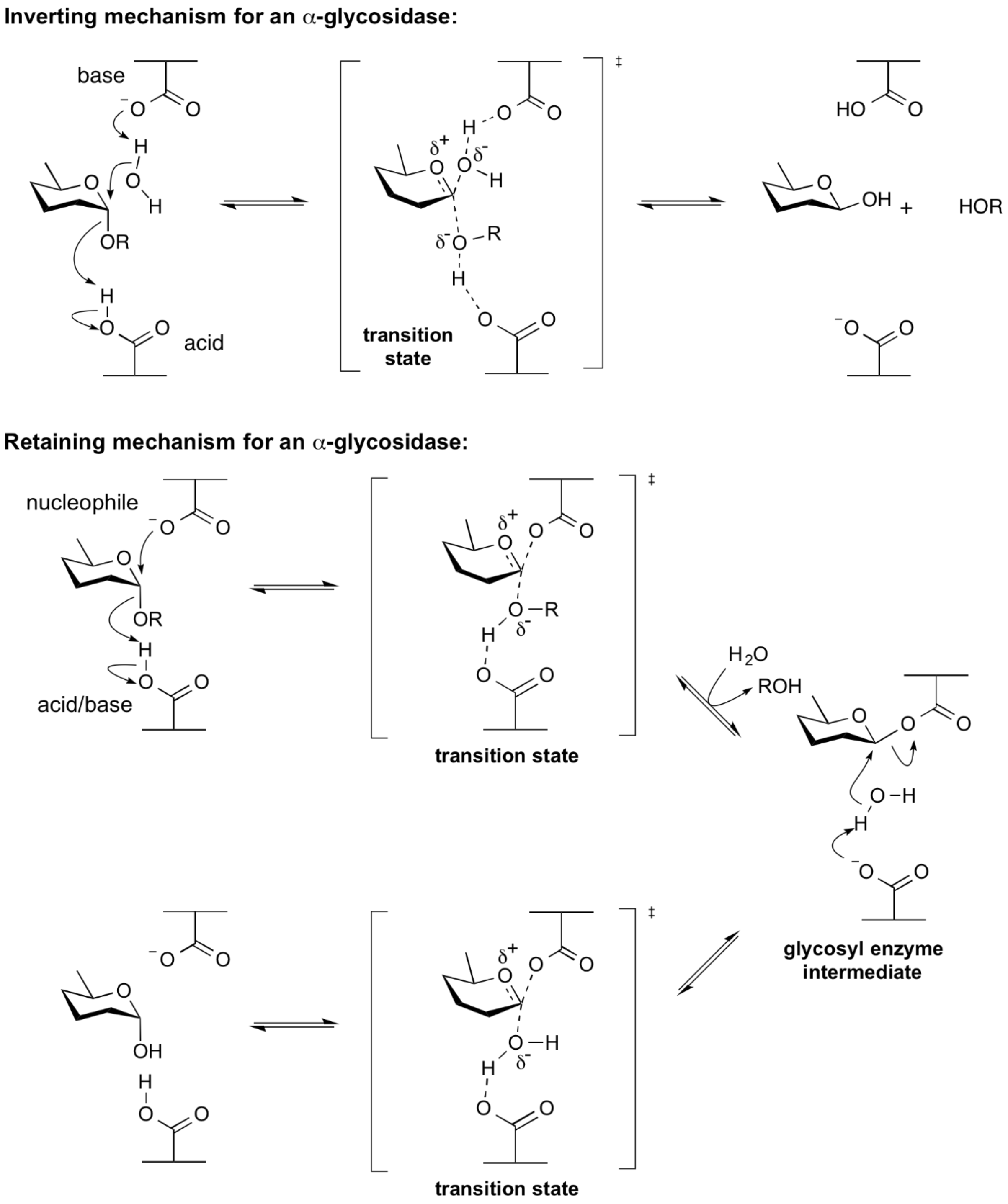


Figure 3.1. Schematic illustration of endo- and exo-activity (A), and subsite designation (B). Exo-acting enzymes cleave the substrate from the end of the substrate and endo-acting enzymes cleave internal parts of the substrate. Subsites are counted from the cleavage site, with negative numbers designating the sites towards the non-reducing end and positive numbers towards the reducing end. Conventionally, the non-reducing end is depicted to the right.

The two most common mechanisms by which GH enzymes act are denoted inverting (Fig. 3.2 A) and retaining (Fig. 3.2 B), which refers to whether or not the anomeric configuration is the same or not after cleavage (52). Although a CAZy family often contains multiple substrate specificities, it has been shown that the mechanism within a family is almost always conserved (39), apart from a few exceptions such as GH97, which contains both inverting and retaining enzymes (53). There are, however, many variations of these mechanisms as well as fundamentally different ones utilized by GH members (54).

Inverting GH enzymes typically use a one-step single-displacement mechanism where two amino acids, normally glutamic or aspartic acids, function as a general acid and a general base. The amino acids are typically located around 10 Å apart, providing enough space for a water molecule to participate in the reaction (50).

Retaining enzymes instead use a two-step double displacement mechanism. The reaction involves two amino acids, again usually glutamic or aspartic acids, which acts as an acid/base and a nucleophile, respectively. These residues are closer together, typically around 5.5 Å apart, which prevents water from coming between the amino acids and the substrate until the second step when the water molecule hydrolyses the covalent adduct (glycosyl-enzyme) intermediate, releasing it from the enzyme (52). Some retaining GH enzymes can have transglycosylase activity; in those cases a glycoside is acting as an acceptor instead of the water in the second step, forming a bond with the covalent adduct (55). An example of a family with transglycosylase activity is GH70, which includes enzymes able to synthesize different kinds of glucans, including α-1,3-glucan, alternan and dextran, with sucrose as a donor (56).



Inverting mechanism for an α-glycosidase:

Retaining mechanism for an α-glycosidase:

Figure 3.2. Mechanisms of glycoside hydrolases. (A) Mechanism of an inverting α-glycosidase. The general base makes a nucleophilic attack on the water molecule which in turn hydrolyses the glycosidic bond, inverting the anomeric configuration. **(B)** Mechanism of a retaining α-glycosidase. The nucleophile attacks the anomeric centre creating a covalent adduct glycosyl enzyme intermediate and a deprotonated acid/base. In the second step the deprotonated carboxyl acts as a base and assists a water molecule in the hydrolysis of the intermediate, creating the hydrolysed product with a net retention of the anomeric configuration. Figure adapted from figures on the Cazypedia page on Glycoside hydrolases under an open access licence (57). Accessed 2026-01-07.

3.3 Carbohydrate-binding modules

The CAZy database also contains a class of non-catalytic proteins called carbohydrate-binding modules (CBMs), of which there are currently 107 families. CBMs are discrete protein domains that are appended to cazymes and facilitate binding of the protein to a saccharide. There are frequently several CBMs in a protein, and these may belong to the same or different families. Usually, the CBM domain binds to the target substrate of the connected cazyme, but there are also cases when CBMs have been shown to bind other targets which are typically assumed to be in the vicinity to the catalytic domain's substrate, for example other polysaccharides in plant cell walls than those targeted by the connected catalytic domain (58). Some CBMs are closely related to lectins, which also promote carbohydrate binding but are not appended to catalytic modules. However, since the CAZy classification is sequence based, lectins may be classified into a CBM family. As with cazymes, CBMs are classified into families based on their sequence similarity, resulting in similar structures within the family. CBM domains has so far been shown to fall into seven different fold families, the most common being the β-sandwich and the others being β-trefoil, cysteine knot, OB fold, hevein fold, hevein-like fold and unique folds (59).

CBMs can be classified into three types based on what kind of binding site towards the target they possess (60, 61) (Fig. 3.3). Type A CBM domains bind planar polysaccharide surfaces, such as the crystalline planes of cellulose and chitin, through aromatic amino acids oriented in a planar configuration allowing hydrophobic and CH-π interactions with the substrate. Type B CBMs have a binding cleft, that allows for binding along and around the glycan chain in an endo-like manner. Type C CBMs instead form shorter binding pockets, binding the end of the glycan in an exo-like manner. Type B and type C CBMs are structurally very similar, and different members of the same CBM family may be classified as either binding type.

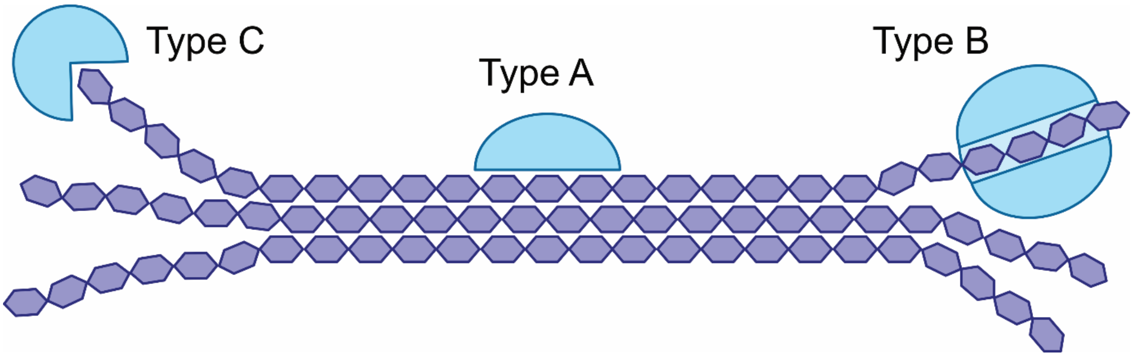


Figure 3.3. Schematic depiction of different types of CBMs. Type A binds planar, crystalline surfaces, type B internal portions of the glycan, and type C saccharide ends.

Chapter 4: Strategies for discovery of novel functionality

There are several strategies that can be employed in order to expand the knowledge about a target enzymatic activity. In this thesis, I divide different strategies into two broad categories: building upon what is already known about such enzymes, or looking for a function and trying to associate it with an enzyme (62).

The first category of strategies could for example utilize the sequence or structure of a characterized enzyme with the desired activity, or a closely related one, and explore the diversity among similar but slightly different enzymes (63). It could also include engineering strategies, such as directed evolution or rational design, where a characterized enzyme would be the starting point subjected to changes to improve or alter its functionality (64).

The second category of strategies often involves some kind of screening to find an activity, followed by identification of the enzyme associated with it. This can result in the discovery of both completely new kinds of enzymes and members of previously known families. Screening could be both culture-dependent, relying on the growth of an organism and its enzyme production, or culture-independent, exploring metagenomes or metaproteomes of a complex sample directly (42, 62, 65).

There is relatively little known about α-1,3-glucanases. There are two enzyme families where α-1,3-glucanase activity have been found, GH71 and GH87, but both families are relatively unexplored. At the start of my PhD, there was just a handful of characterized enzymes in each family, and their mechanisms were not entirely elucidated. There are also cases of α-1,3-glucanases purified from species which do not encode any genes for GH71 or GH87 enzymes, such as *Pseudomonas* species (66, 67), indicating that there are so far undiscovered enzyme families with α-1,3-glucanase activity, or enzyme families to which this activity has not previously been associated.

In my work, I employ two different strategies, exploring the known enzyme families and looking the activity of interest in nature and trying to identify the cause behind such an observation. In this chapter, I present an overview of the foundation of knowledge already existing for each approach and my contributions to the field based on the work presented in my articles.

4.1 Exploring diversity within known families

4.1.1 GH71

The first enzyme for which α-1,3-glucanase activity was found, and linked to a specific protein, was isolated from *Trichoderma viride* in 1969 (68). The secretion of this protein was induced by the presence of α-1,3-glucan extracted from *Aspergillus niger,* and the enzyme was shown to be active on oligo- and polysaccharides containing α-1,3-linkages. Since the amino acid sequence of this enzyme was not known, it was not until 2000 that GH71 was established based on the recombinantly produced enzymes originating from *Trichoderma harzianum* and *Penicillium purpurogenum* (69). Earlier studies focused on how the GH71 enzymes affected the fungi's physiology, and these showed that α-1,3-glucan can function as an energy storage molecule in *A. nidulans* that gets degraded by α-1,3-glucanase activity during carbon starvation (29). In *Trichoderma* fungivores, such as *T. harzianum* and *T. asperellum*, the enzyme was instead shown to target other fungi's cell walls as part of their mycoparasitic lifestyle (70, 71). In yeasts, GH71 enzymes have been shown to have a different role, where the α-1,3-glucanases are active on the encoding organism's own cell wall, and such activity were shown to be necessary for complete cell separation during cell division (72, 73).

Analysis of the phylogenetic diversity of GH71 enzymes, as is shown on CAZy (39) and **Article I**, revealed their presence in fungi and bacteria in about equal amounts, and even occasional members were found in plantae and rotifers (Fig. 4.1). Despite being known for a long time, surprisingly few enzyme members of the family have been characterized to date. Most characterized enzymes have been from filamentous fungi such as *Trichoderma, Talaromyces* and *Aspergillus* genera, a few from yeasts belonging to *Schizosaccaromyces* genera (32, 73) and one from the dimorphic fungus *Paracoccidioides brasiliensis* (32), but as of yet none from bacteria.

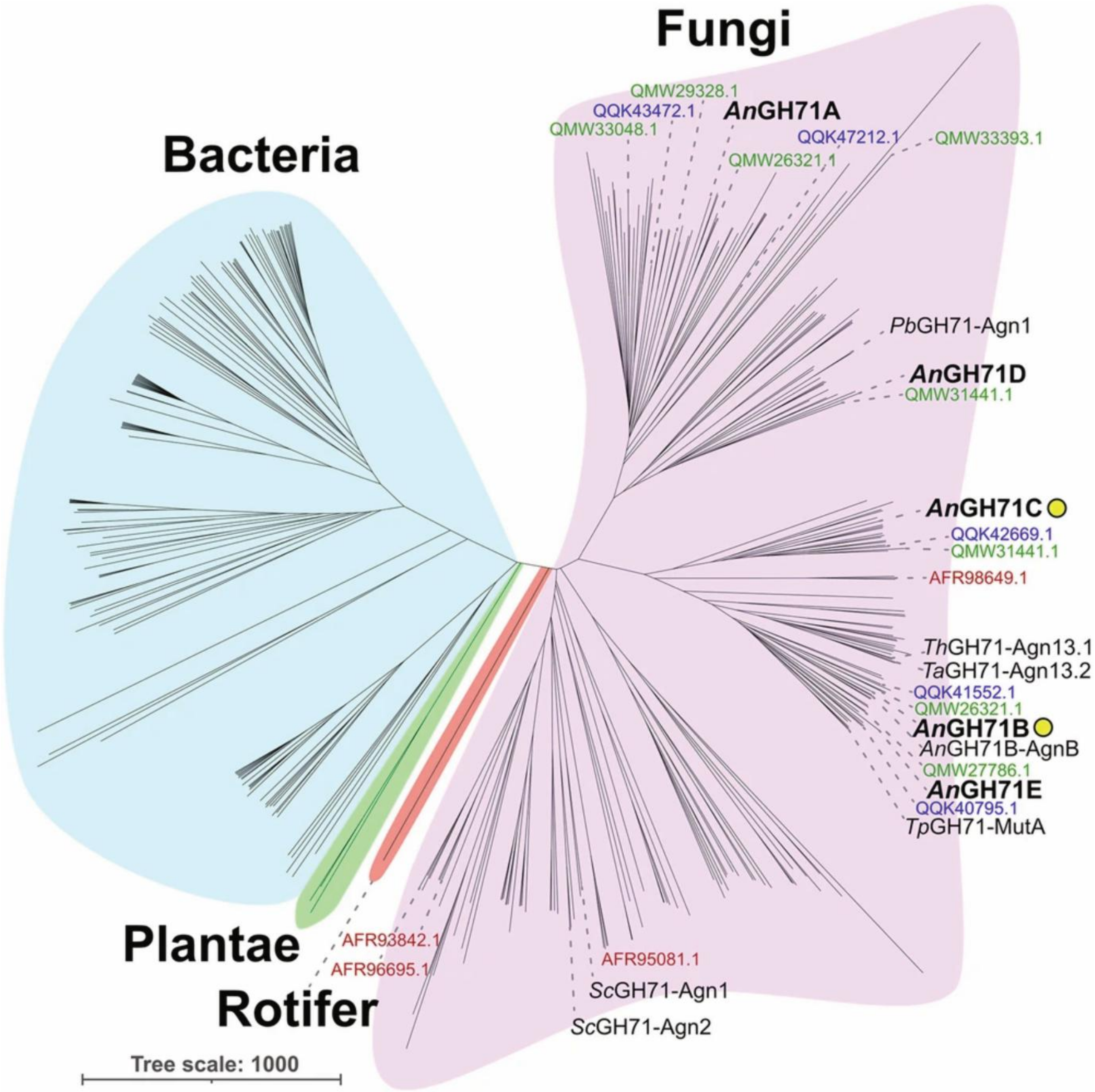


Figure 4.1. Phylogenetic tree of GH71. (Article I (74)**).** Characterized members are marked with name or GenBank identifiers. The enzymes from *A. nidulans* are marked in bold and the enzymes studied in **Article I** with yellow circles. Examples of other species encoding multiple GH71 genes have colored Genbank IDs, *Cryptococcus neoformans* var. grubii H99 (red), *Penicillium digitatum* PdW03 (blue), and *Aspergillus flavus* NRRL3357 (green).

Structure and mechanism of GH71 enzymes – results related to Article I

At the start of my PhD there was no published structure of any GH71 enzyme, limiting mechanistic insights. The project was initiated by the solving of the first crystal structure of a GH71 enzyme by Dr. Scott Mazurkewich in our group, and at the start of my PhD studies I joined him in the biochemical characterization of two enzymes from *A. nidulans*, *An*GH71B and *An*GH71C, in order to couple the structural and biochemical data to gain insights in the mechanism of the enzymes.

Curiously, the genome of *A. nidulans* encodes five different GH71 enzymes from across the phylogenetic tree. While we aimed to produce all of these, we only managed to produce two in soluble form, despite employing multiple expression strategies using chaperone strains in *E. coli* and *Pichia pastoris* as a fungal expression host. The enzymes characterized in our study, *An*GH71B and *An*GH71C, have previously been shown to prevent the accumulation of α-1,3-glucan when overexpressed, and thus been deemed functional α-1,3-glucanases (75). Conversely, a knockout strain of *An*GH71C was shown to have an increased amount of α-1,3-glucan, supporting the notion that *An*GH71C has significant α-1,3-glucanase activity (76). Based on sugar release analyses from the tested substrates, our study confirmed that both enzymes were active α-1,3-glucanases without detectable activity on any of the other tested substrates, including the similar polysaccharides alternan, dextran and starch. Surprisingly, we found that their activity was limited to oligosaccharides and the soluble portions of the mainly insoluble α-1,3-glucan.

The structure of *An*GH71C (Fig. 4.2) clearly showed the substrate binding cleft adopting a conformation which perfectly accommodates the zigzag-like structure of α-1,3-glucan. In this cleft, seven sugar-binding subsites could be identified (-4 to +3) and the possibility of three additional subsites (+4 to +6) were suggested by a bound nigerotriose ligand, although the lack of enzyme-substrate interactions at the latter sites makes the conclusion uncertain. Based on the structure and conservation in other GH71 enzymes, as well as GH99 enzymes which share similar overall structure, Asp265 and Glu268 were identified as likely catalytic residues. To confirm the identity of the catalytic residues, we substituted them individually with alanine which reduced the activity 200-15,000 fold compared to the wild type (Table 3, **Article I**), supporting this hypothesis.

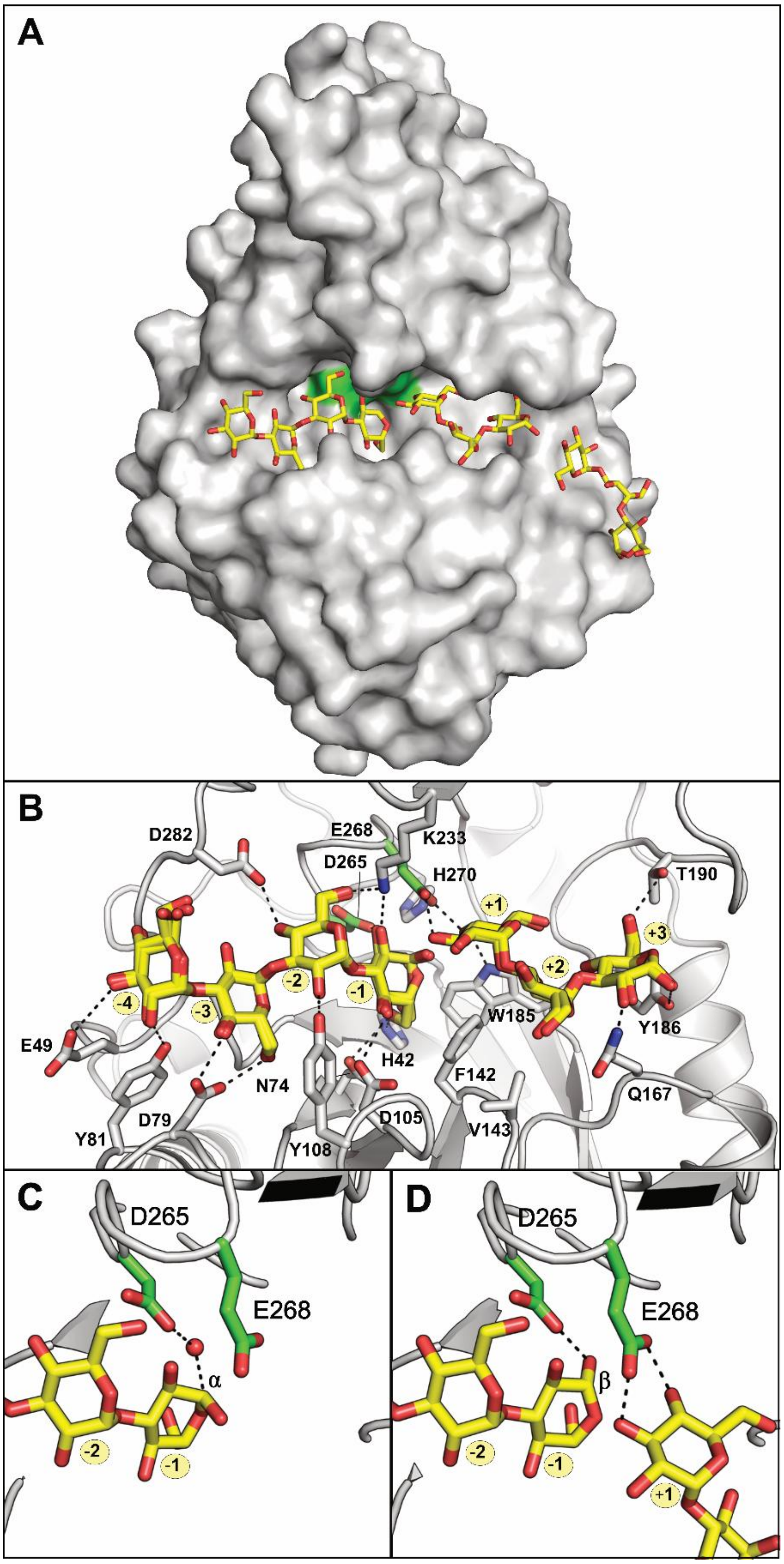


Figure 4.2. Structure of *An*GH71C with ligands (PDB: 9FNH). **(A)** surface. **(B)** zoom in on substrate binding cleft. **(C)** Active site with a nigerose ligand with the reducing end hydroxyl in α-configuration, mimicking the enzyme-substrate interaction, with a water molecule positioned between the anomeric carbon and Asp265. **(D)** Active site with a nigerose ligand with the reducing end hydroxyl in β-configuration, positioned as after a cleavage with inverting mechanism. Adapted from Fig. 4, **Article I**.

A previous study showed an inverting mechanism for a GH71 enzyme from *T. harzianum* using NMR (77). Our structure and corresponding analysis of *An*GH71C indicated the same mechanism for this protein. In different chains of the structure, ligands where the reducing end of the ligand is in both α- and β-configurations were found. When the reducing end of the ligand is in α-configuration, as it would be internally in a longer substrate, a water molecule is present between Asp265 and the anomeric carbon (Fig. 4.2), while when the ligand end is in β-configuration this position is occupied by the C1 hydroxyl group, as it would be in an inverted product. To verify the mechanism, we studied the product accumulation over time using 1D ^{1}H NMR. Using nigerotetraose, the products for both enzymes were nigerotriose and glucose. Initial build-up of the β-anomer of nigerotriose, followed by a build-up of the α-anomer over time, indicates the product is the β-anomer which gets converted to the α-anomer over time due to mutarotation. That the initial products were mainly β-nigerotriose and a mix of α- and β-glucose indicates that the enzymes cleave the substrate with three glucose units in the negative subsites.

To investigate the mode of action of the enzymes, i.e. whether the enzymes act in an endo- or exo-fashion, we studied the product profiles using different oligosaccharides as substrates, and the same oligosaccharides where the reducing end had been reduced to the corresponding alditols using borohydride. The minimal substrate for both enzymes was nigerotetraose, but with some trace activity on nigerotriose. However, *An*GH71B showed decreasing specific activity with higher degree of polymerisation (between nigerotetraose - nigerohexaose), while the opposite was true for *An*GH71C. The product profiles were also different, *An*GH71B produced glucose, nigerotriose and a smaller amount of nigerose regardless of substrate while *An*GH71C produced varying amounts of glucose, nigerose, nigerotriose and nigerotetraose (Fig. 2, **Article I**). When using the reduced nigerohexaose as a substrate, *An*GH71B only produced the reduced form of nigerose, but not of glucose or nigerotriose, indicating subsequent cleavage from the reducing end, i.e. exo-activity. On the other hand, *An*GH71C produced both reduced nigerose and nigerotriose, indicating a random internal cleavage, i.e. endo-activity (Fig. 4.3).

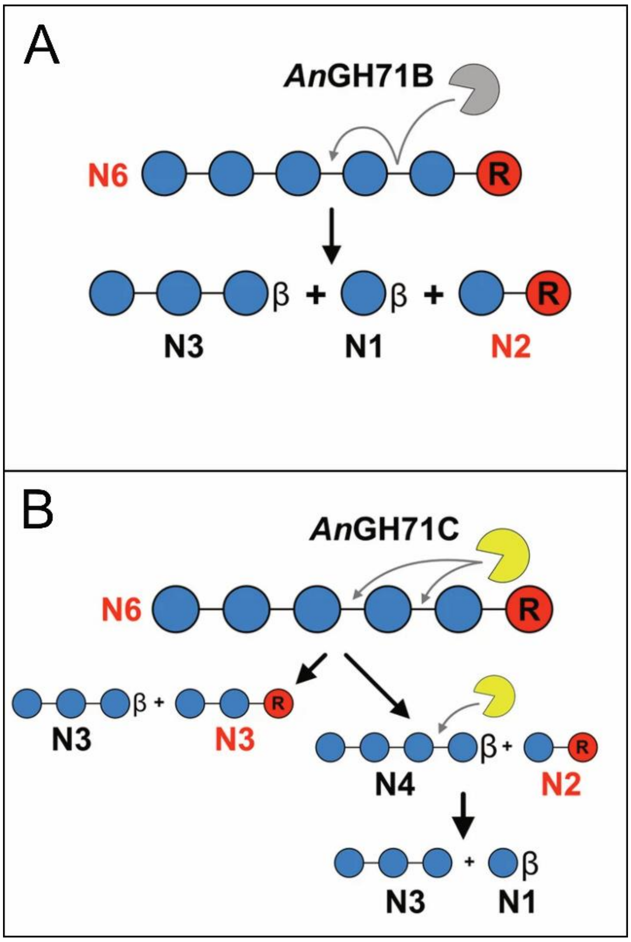


Figure 4.3. Proposed cleavage reactions of *An*GH71B (A) and *An*GH71C (B). *An*GH71B cleaves the substrate from the reducing end, subsequently releasing glucose. *An*GH71C cleaves the substrate internally releasing a mixture of products. Both enzymes require at least three glucose units at the non-reducing end to be recognised in the negative subsites. Figure adapted from Article I.

Another potential mode of action the enzymes could have is endo-processive, i.e. cleaving the substrate internally and then continuing its activity from that point. This kind of activity was suggested for the GH71 enzyme MutAp from *T. harzianum* (77) and could be the one used by *An*GH71C as well, though this is difficult to determine with substrates with as short chain length as the ones used in our study. Conversely, *An*GH71B is unlikely to be endo-processive since we don't see any reduced nigerotriose among the products, indicating the enzyme doesn't cleave the substrate at a random internal position.

During the review of our article, another crystal structure of another GH71 enzyme was published, this one from *S. pombe* (78). This study suggested two amino acids corresponding to the two investigated in our study, and one additional aspartate on the opposite side of the substrate binding cleft (Asp69 in 8YFH) as important for α-1,3-glucanase activity based on substitution studies. However, the ligands in their structure were between the +2 and +7 sites, and not published on PDB, making conclusions around the mechanism difficult. Judging from our structures with ligands, the corresponding Asp does not seem involved in catalytic activity due to its distance from the cleaved glycosidic bond.

GH71 enzymes are variably found to be exo- or endo-acting (70, 71, 77, 79, 80), including the subjects of our study, *An*GH71B and *An*GH71C. This difference is likely due to subtle changes in the active cleft, *An*GH71B has an extended loop close to the active cleft compared to *An*GH71C, possibly blocking random access to internal parts of the oligosaccharide chain. A difference in activity also motivates the purpose for organisms to have multiple copies of GH71 enzymes, presumably having complementary biological roles.

Summary and outlook of section

Despite having first been identified decades ago, there is still much to learn about GH71 enzymes. Our work in **Article I** provided answers to several knowledge gaps regarding this family, solving the first protein structure with ligands and elucidating the mechanism. However, some interesting questions remain. *An*GH71B and *An*GH71C surprisingly seemed only to be active on soluble parts of α-1,3-glucan. It is difficult to determine whether the same was true in previous studies of these specific enzymes, since they were studied either through knockouts in *A. nidulans* or on polysaccharides extracted from fungi which likely contains both soluble and insoluble fractions (75, 76, 81). Since other GH71 enzymes have been reported to be active on insoluble substrates (69, 70, 82) there is likely a diversity within the family regarding this ability. Overall, GH71 seems to contain a lot of variability among its members, having both endo- and exo-activities. Because many fungi encode several GH71 enzymes, such differences can likely be attributed to varying biological roles. Future studies may show if the functional variations can be derived from structural differences.

4.1.2 GH87

As for GH71, only a few members from GH87 have been characterized to date. However, at the start of my PhD, several crystal structures had recently been solved, further advancing our understanding of these types of enzymes. GH87 enzymes are exclusively present in bacteria. Of the few characterised enzymes from this family, most come from the genus *Paenibacillus* (83, 84) but also *Streptomyces* (85)*, Niallia* (86), and *Flavobacterium* (87). All of the characterized enzymes have been shown to be α-1,3-glucanases, except for Myd from *Streptomyces* sp. J-13-3 which was shown to be a mycodextranase, cleaving the α-1,4-linkages in nigeran (α-1,3/α-1,4-glucan) but not pure α-1,3- or α-1,4-glucan (88).

Three structures of GH87 enzymes have been solved (85, 86), including one with multiple ligands (89). The catalytic domain, i.e. what is recognised as belonging to GH87, consists of an N-terminal β-sandwich fold module, somewhat similar to domains from CBM6 and CBM35, and a β-helix fold module which contains the active site cleft. Many aromatic residues contribute to sugar binding in the active cleft, forming 8 subsites from -4 to +4. The subsites occupied in the structure in Fig. 4.4 are -3, -2, +3, and +4. The role of the N-terminal β-sandwich domain is not entirely clear. Compared to CBM35 domains, it has an extended loop which contributes to the side of the active cleft (89), and may contribute to binding of the substrate.

The combination of structural investigation, conservation within the family, comparisons with enzyme families that have a similar fold (GH28 and GH49), and substitution studies have elucidated the catalytic residues as three aspartates in the centre of the enzyme (Asp1045, Asp1068 and Asp1069 in the structure of Agl-FH1from *Paenibacillus glycanilyticus* FH11, PDB: 6K0N), with Asp1068 acting as the general acid and either Asp1045 or Asp1069 acting as general base in the inverting mechanism (89). To the best of my knowledge, all characterized GH87 enzymes have been reported to be endo-active (21, 39, 84, 85, 89, 90). However, in the similar families GH28 and GH49, which, as mentioned, have similar folds and conserved catalytic residues, both endo- and exo-acting enzymes have been described (39). It should be noted however that the active clefts in GH87 enzymes have significant structural differences compared to GH28 and GH49 enzymes (89), and so the comparison might not be completely applicable.

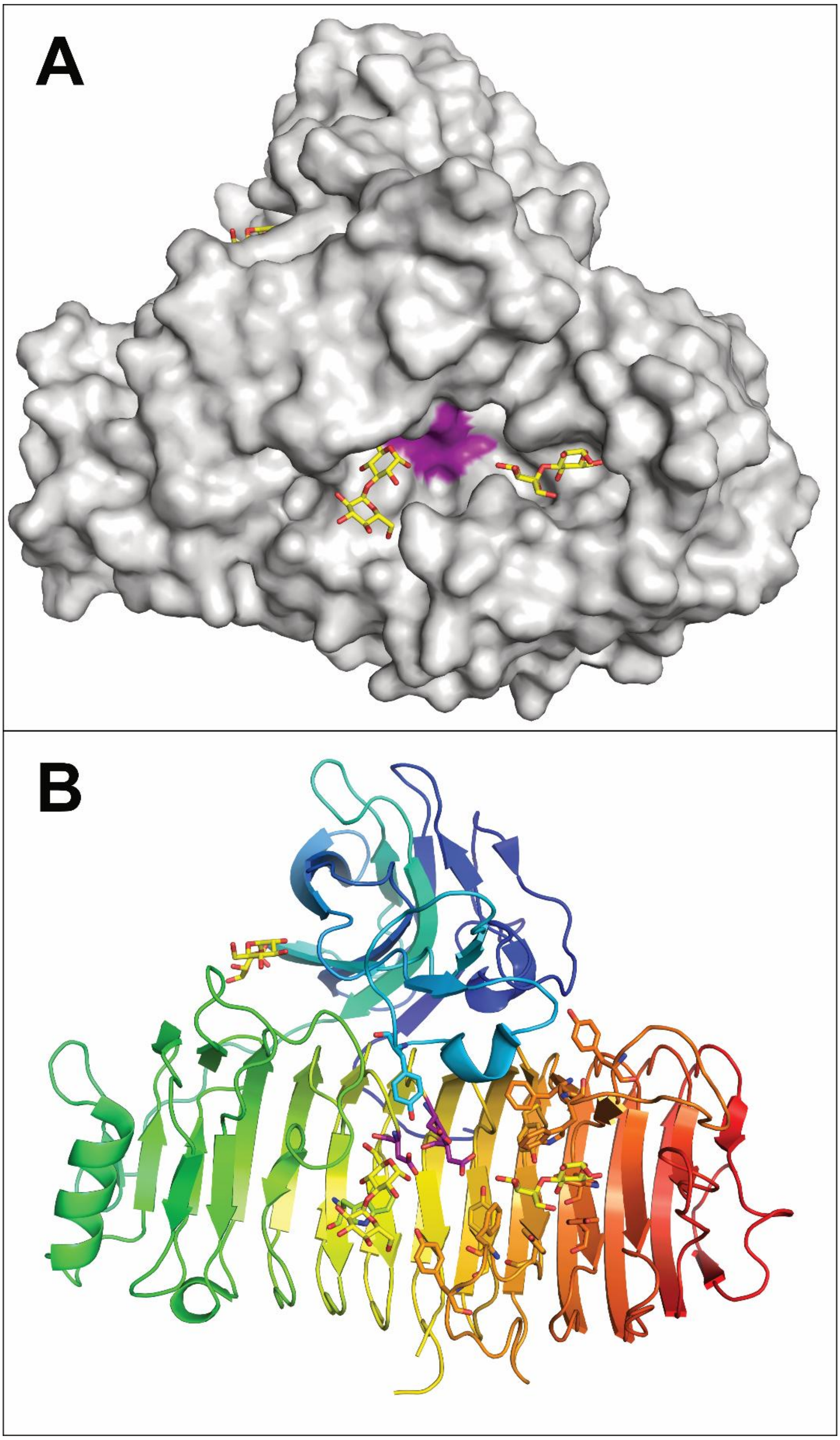


Figure 4.4. Crystal structure of the GH87 enzyme CatAgl-FH1 from *P. glycanilyticus* (PDB: 6K0N) (89). **(A)** Protein surface with the catalytic residues marked in purple and nigerose ligands shown as yellow sticks. **(B)** Cartoon of the protein coloured in a rainbow spectrum from the N-terminus (blue) to the C-terminus (red), with nigerose ligands shown as yellow sticks and the catalytic aspartate residues in purple. Aromatic residues contributing to binding are shown as sticks.

In my studies, I have demonstrated the α-1,3-glucanase activity of the enzyme *Fj*GH87 from *F. johnsoniae* on both α-1,3-glucan and alternan (**Article II**), indicating that the enzyme can accommodate α-1,6-linkages to some degree even if they are not cleaved. *Fj*GH87 was clearly active on insoluble α-1,3-glucan and could cleave nigerotetraose as the minimal substrate, indicating similar activity to previously characterized GH87 enzymes (87, 90).

4.1.4 Carbohydrate-binding modules targeting α-1,3-glucan

CBM24

The only CBM family listed in CAZy where mutan or α-1,3-glucan-binding ability has been demonstrated is CBM24. Though CBM24 domains have been shown to bind α-1,3-glucan in two separate proteins—GH71 enzymes from *Trichoderma harzianum* and *Talaromyces purpureogenus* (previously known as *Penicillium purpurogenum*)—the family has not been studied in greater detail. Therefore there is no information about important binding residues or the biological role(s) of such domains (69). CBM24 is only present in eukaryotic organisms and these CBMs have mostly been found appended to GH71 domains (39). CBM24 domains are very small, often seemingly containing a long linker between them and the catalytic domains. So far, these domains have only been detected in proteins from filamentous fungi and not in any yeast GH71 proteins (73). Despite potential interesting applications for α-1,3-glucan-binding proteins, and how knowledge about them could increase the understanding of connected α-1,3-glucanases, they have only been studied in a single publication to my knowledge (69). This article led to the establishment of both CBM24 and GH71, and while other studies have mentioned and theorized around their presence, no further experimental studies have been conducted. Therefore, our current understanding of these protein domains is far from complete.

Other CBM-like domains connected to α-1,3-glucanases

Suyotha et al. (91) characterized a GH87 enzyme from *Niallia circulans* Agl-KA, and its appended domains, and found that three of them individually promoted weak binding to α-1,3-glucan. When these domains were joined together, it further increased the binding efficiency which enabled the creation of a new construct together with a fluorescent protein which could be used for efficient visualisation of fungal cell walls. The binding domains were two discoidin domain-containing proteins, called DS1 and DS2, and a CBM6 domain, referred to as CB6. Other members of CBM6 have been shown to bind very different carbohydrates, including hemicelluloses, β-glucans and agarose (92), and indeed CB6, as well as DS1 and DS2 bound weakly to xylan in addition to α-1,3-glucan (91). Though specific binding residues in CB6 have not been studied for their role in binding α-1,3-glucan, important residues in CBM6 have been studied for other domains. CBM6 is known to be a family of high binding variability and, as is the case with CBMs in general, slight changes in important residues can significantly alter the binding specificity (92). DS1 and DS2 are fairly similar to each other (41 % sequence identity), and though they have not been categorized in a designated family, similar protein domains appear together with other GH87 domains from both *Paenibacillus* and *Streptomyces* (83, 89, 93), including the GH87 enzymes detected in our proteomic study, for which the binding domains are classified as CBM32 (see section 4.2.2 and **Article IV**). A GH87 enzyme from *P. glycanilyticus*, Agl-FH1 had a similar domain architecture as Agl-KA, but the domain between the discoidin domains was identified as a CBM35 domain and not as a CBM6 domain (89). CBM35 is a family containing variable binding specificities, including to α-glucans such as α-1,4- and α-1,6-linked oligosaccharides (94–96).

It is interesting to note that CBM6 and CBM35 are distantly related (97), and together with CBM32 they all have a β-sandwich fold. All three families display highly variable substrate specificities due to the binding sites originating from variable loop regions of the proteins, and they can be found appended to many different kinds of catalytic domains (98, 99, 94, 100). It would be interesting to investigate whether any similarities across the families could be found among those domains that bind α-1,3-glucan.

Discovery of CBMXXX – results related to Article II

During my investigation of the GH87 enzyme from *F. johnsoniae, Fj*GH87, the characterization of a very similar enzyme, Agl-EK14 from *Flavobacterium* sp. EK14, was published by Takahashi et al. (87). This enzyme was verified to be an active α-1,3-glucanase, like *Fj*GH87. However, in my studies I had discovered that the full-length, multi-modular enzyme seemed to work better than the isolated catalytic domain (see **Article II**, Fig 2). Interestingly, none of the additional domains belonged to a known CBM family. The domain architecture of *Fj*GH87 (Fig. 4.5) was identical to that of Agl-EK14, but only the full-length Agl-EK14 protein had been studied (87). We hypothesized that one of the additional domains were responsible for increasing the efficiency of the enzyme by facilitating substrate binding and decided to test this hypothesis by characterizing its potential as binding domain.

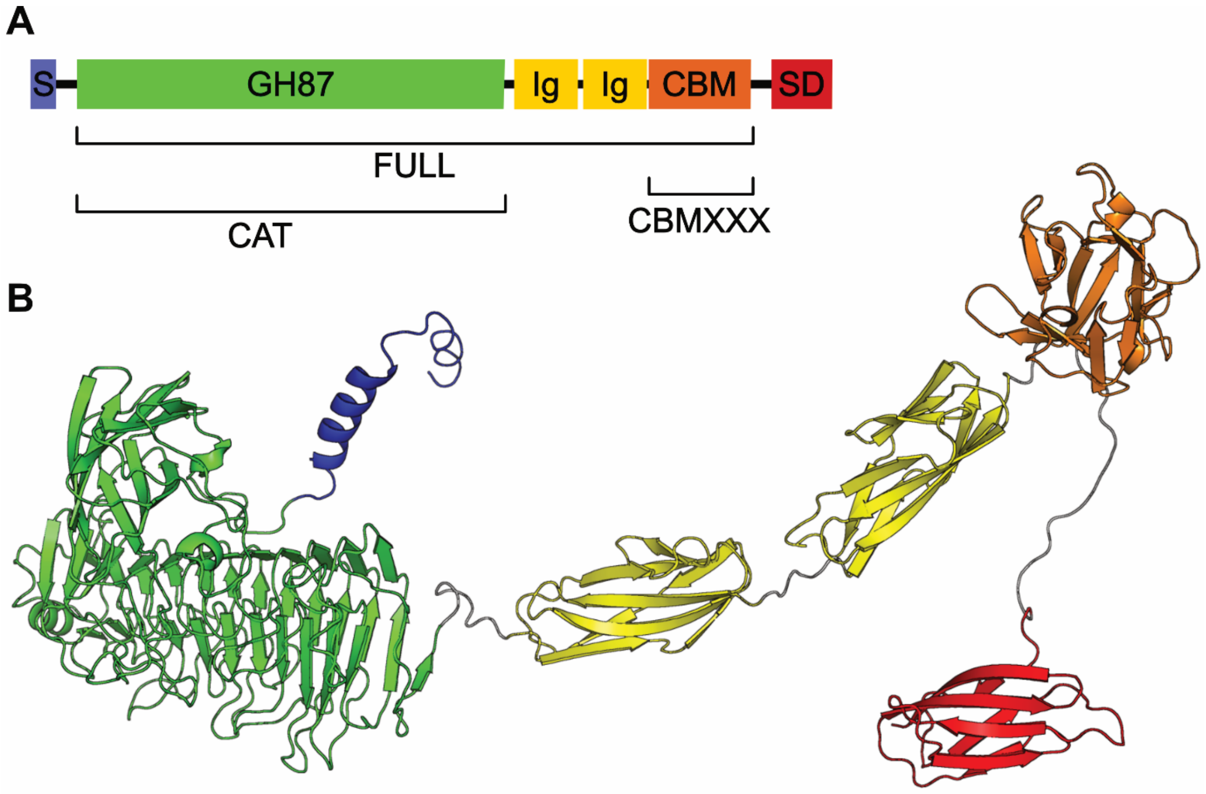


Figure 4.5. Domain architecture of *Fj*GH87. (A) Schematic representation of the domain architecture and the constructs created. The different domains are S: signal peptide, GH87: the catalytic GH87 domain, Ig: Immunoglobulin-like domains, CBM: the putative binding domain, SD: C-terminal sorting domain. The constructs are: FULL: the full-length protein without signal peptide and sorting domain, CAT: the catalytic GH87 domain, CBMXXX: the putative binding domain. **(B)** AlphaFold model with the angles of the linker regions between the domains adjusted for clear visualization. Figure from **Article II.**

To investigate the function of the putative binding domain, it was cloned and produced separately. Binding to insoluble polysaccharides was assessed through pulldown assays, i.e. where the protein was incubated with insoluble polysaccharides, the mixture centrifuged to pellet polysaccharides and bound proteins, and potential decrease in protein concentration in the supernatant visualized through SDS-PAGE. This assay (Fig. 4.6) showed binding to α-1,3-glucan, alternan and potentially weakly to nigeran but not any of the other tested polysaccharides.

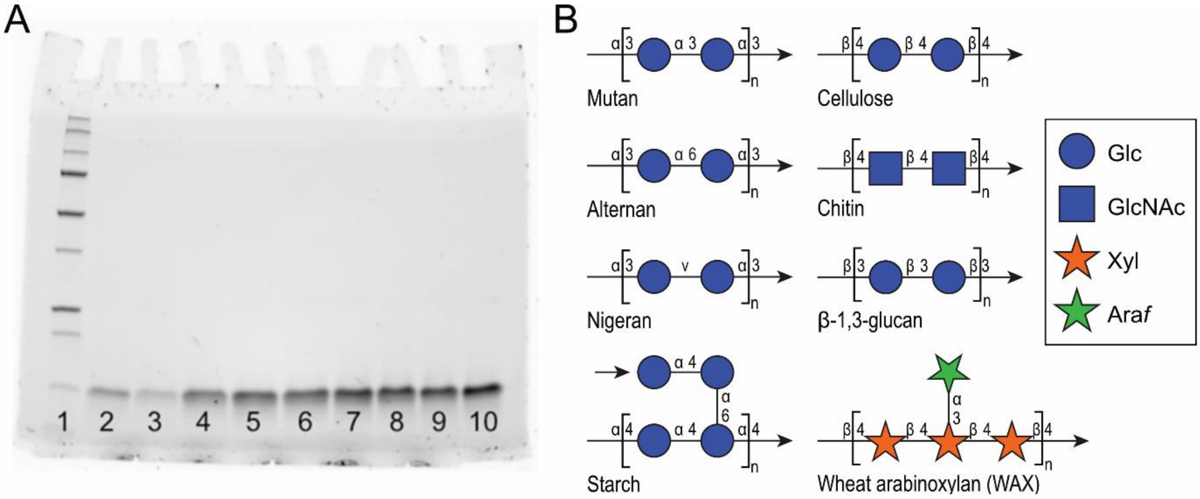


Figure 4.6. **(A)** Pulldown assay of FjCBMXXX$_{\text{GH87}}$. 1: molecular weight ladder, 2: α-1,3-glucan, 3: alternan, 4: nigeran, 5: starch, 6: cellulose, 7: wheat arabinoxylan , 8: chitin, 9: yeast β-glucan, 10: negative control. **(B)** Composition and linkages of the tested polysaccharides. Figure from **Article II**.

Having confirmed that the domain indeed facilitated binding, we investigated its sequence and (predicted) structure more carefully. Currently this domain is referred to as *Fj*CBMXXX$_{\text{GH87}}$ where “XXX” is a placeholder for the family number that will be assigned by CAZy upon acceptance for publication of the article. To investigate the prevalence of this type of domain in other proteins, I performed a BLAST search against the ClusteredNR database (101). The analysis showed that homologs to *Fj*CBMXXX$_{\text{GH87}}$ do not fall into any other CBM families but seem to form their own group of sequences. To visualize the diversity of this putative family, the top 250 hits from the BLAST search were used to construct a phylogenetic tree, with colour-coding of the phyla of the encoding species and with the domain architecture of the full-length protein added as an additional layer outside the tree (Fig. 4.7 A). CBMXXX domains were found to be present exclusively in bacteria, many in Bacteroidota as the query, but also in Pseudomonadota, Bacillota, Actinomycetota, and Myxococcota, with a few occurrences in other bacterial phyla. By studying the full-length proteins in which CBMXXX domains occur, I learned that they are mainly associated with GH87, GH13, GH16 and GH99 domains, and that they can be present both at the N- and C-terminal sides of the catalytic domain (Fig. 4.7 B). The co-occurrence of CBMXXX domains with this wide distribution of catalytic domains indicates a variety of possible binding abilities.

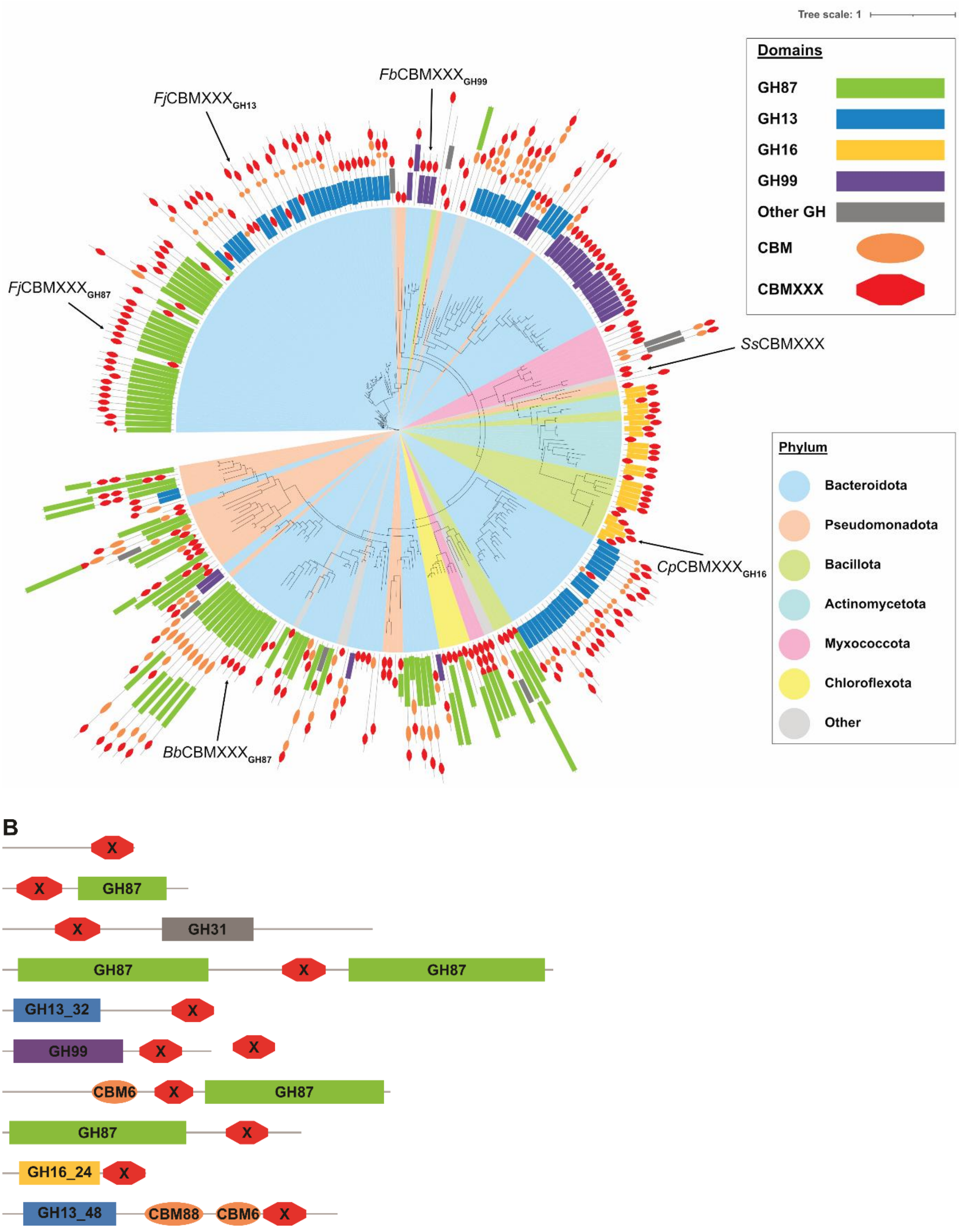


Figure 4.7. (A) Phylogenetic tree of the aligned 250 top hits of a BLAST search with *Fj*CBMXXX$_{\text{GH87}}$ as query. The coloured branches indicate the phylum association. The domain architecture is displayed outside the tree. For a larger version of the figure, see Fig. 5 **Article II**. **(B)** Selection of different domain architectures including CBMXXX domains. CBMXXX domains (red octagons marked with X) occur in proteins together with various catalytic domains or alone, and can be present on both the N- and C-terminal parts of the proteins.

To gain insights into the binding mechanisms of CBMXXX domains, we wanted to study its structure as well. Attempts to crystallize the protein were unfortunately unsuccessful and so an AlphaFold model was instead generated. The domain was shown to have a β-trefoil fold (Fig. 4.8) characterized by the three symmetric repeats of four β-strands (102).

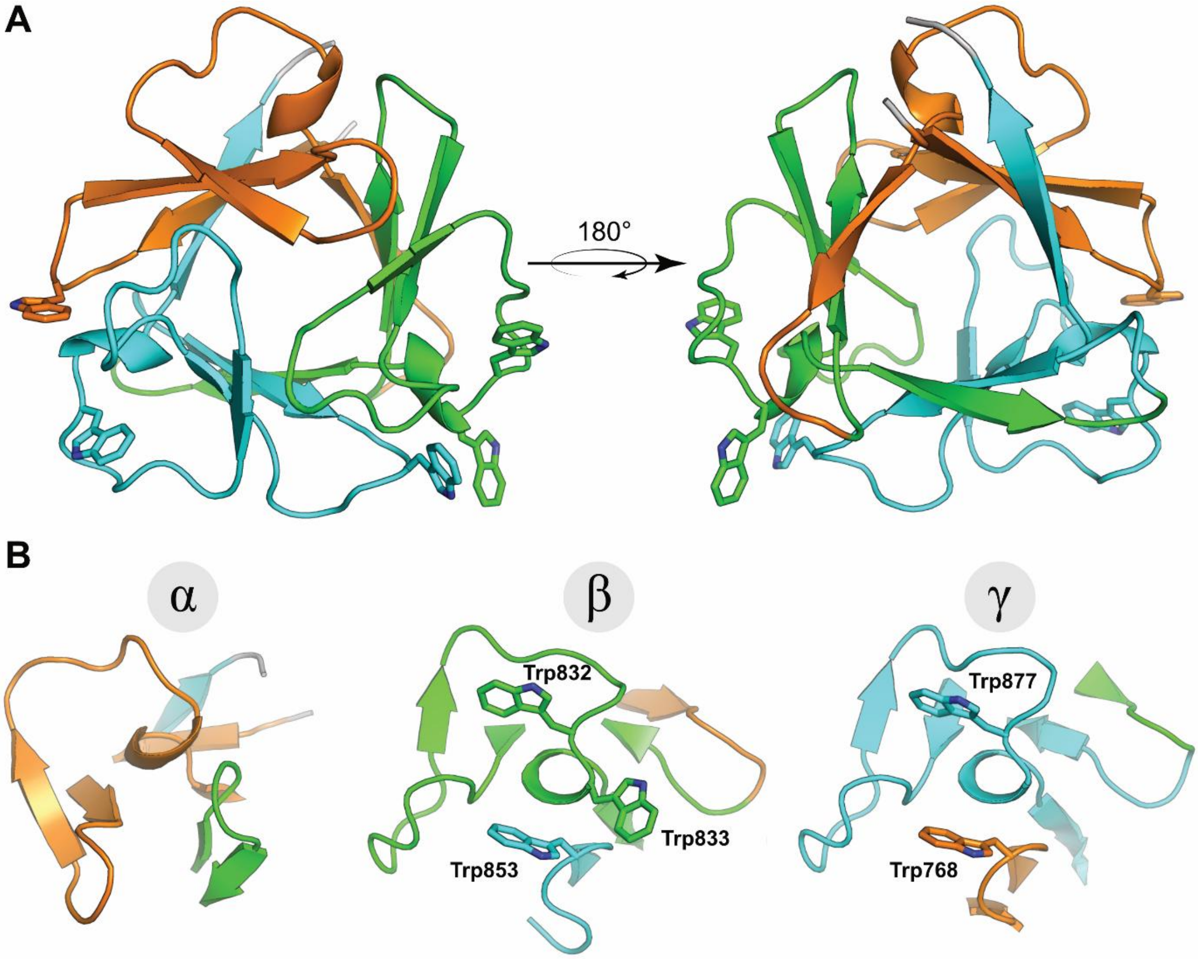


Figure 4.8. AlphaFold model of *Fj*CBMXXX$_{GH87}$ with the putative binding residues showed as sticks. (A) The leaflets are color-coded based on their subdomain designation. **(B)** The individual leaflets are aligned to each other, with putative binding residues labelled.

This type of fold has so far been found in CBM13, CBM42 and CBM92 in CAZy (103–105). While CBMXXX is structurally similar, especially to CBM13 and CBM42, its sequence differs significantly and the positioning of the binding residues is not conserved among the families. Since carbohydrate-binding by CBM domains is most commonly facilitated, at least in part, by aromatic amino acids, we searched for exposed aromatic amino acids that potentially could facilitate binding. Since β-trefoils have a threefold symmetry, with the subdomains often denoted as α-, β- and γ-leaflets, there is potential for three binding sites, one each in these regions (105). We identified three putative binding sites, comprising two and three tryptophans, and two tyrosines, respectively, forming shallow pockets. However, the tyrosine-containing binding site was not at the corresponding position as the other sites and was shown to be non-functional. The tryptophan-containing binding sites were located around the small α-helix of the β- and γ-leaflets, but curiously one of the tryptophan residues originated from the next leaflet (Fig. 4.9). In the case of the γ-binding site this means the binding residues originate from the beginning and end of the protein, unlike in the other β-trefoil families where the binding residues for each site tends to be localized together.

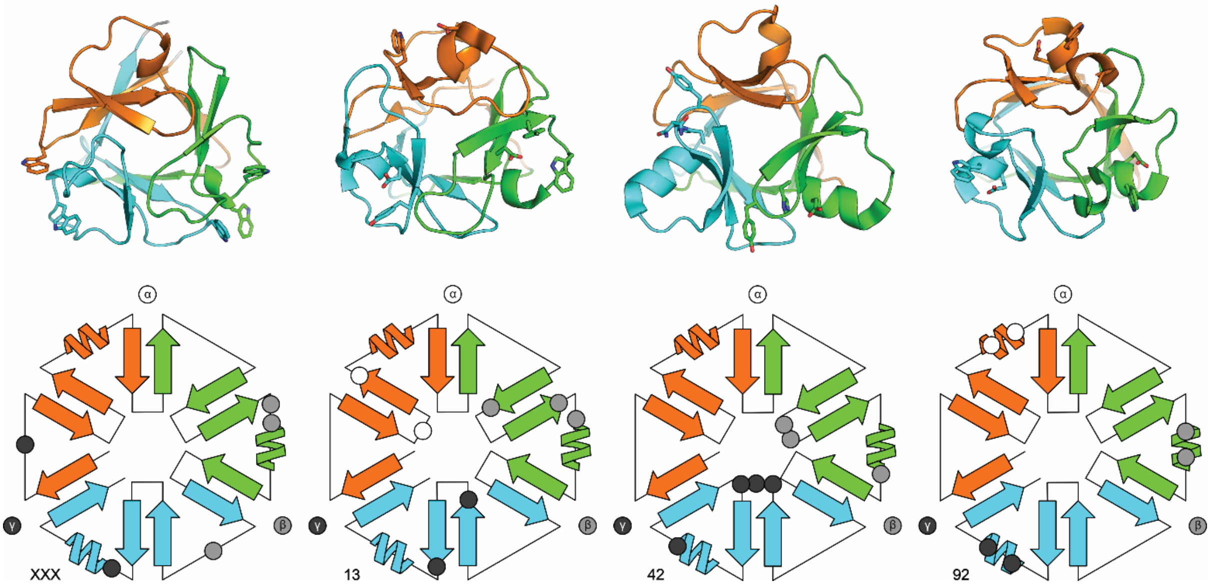


Figure 4.9. Structures and schematic representation of CBM families with β-trefoil fold. Top row: from left to right, AlphaFold model of *Fj*CBMXXX$_{\text{GH87}}$, crystal structures of representatives from CBM13 (PDB: 1KNM), CBM42 (PDB: 9NXI) and CBM92 (PDB: 7ZON). The domain structure is coloured after corresponding leaflet: α-leaflet (N-terminal): orange, β-leaflet: green, γ-leaflet (C-terminal): cyan. Binding residues are shown as sticks. Bottom row: Schematic representation of the same proteins, using the same colour codes. Placements of binding residues are marked with circles, coloured depending on site affiliation.

To verify the function of the putative binding residues, I substituted them with alanine and assessed the binding of the resulting protein variants compared to the wild-type protein, again using pulldown assays. Substitution of the residues in either site abolished binding to both α-1,3-glucan and alternan, confirming that we had correctly identified the binding sites, and in addition showing that both of them were necessary to facilitate binding to the polysaccharides.

We also studied the binding towards oligosaccharides using isothermal titration calorimetry (ITC), which revealed binding to nigero- (α-1,3-linkages), malto- (α-1,4-linkages) and isomaltooligosaccharides (α-1,6-linkages) but not to glucose. When studying the binding site variants, the ITC experiments showed that the substitutions of the binding residues in the β-site abolished or significantly reduced the binding to all oligosaccharides. In contrast, substitutions in the γ-site had no significant impact on the binding towards the oligosaccharides. This is somewhat contradicting the results from the pulldown assays, where the substitutions in either binding site abolished the detectable binding to the polysaccharides. We theorize that this discrepancy indicates that while the β-site binds more strongly to oligosaccharides, the γ-site still facilitates binding, and that the combination of both is needed to bind strongly enough to the polysaccharides for it to be detected in pulldown assays. It is also possible that the regions between the binding sites could have important contributions to the binding of longer oligosaccharides and polysaccharides. This could potentially also explain the varying length preference seen with the differently linked oligosaccharides, in case such regions could accommodate different saccharide linkages varyingly well.

Looking further into other members of CBMXXX, we found that the placement of putative binding residues is generally conserved, but that it is variable which sites have a complete set of putative binding residues, and which aromatic residues comprise them (see Fig. 6, **Article II**). Based on the variability of the catalytic domains to which CBMXXX domains are appended, we expect different substrate specificities within the family. However, investigating the binding ability of *Fj*CBMXXX$_{\text{GH13}}$, which is connected to a GH13 catalytic domain and a maltooligosaccharide-binding CBM26, we found only weak binding to maltopentaose. Whether other CBMXXX domains promote binding to other polysaccharides than α-1,3-glucan, and whether there are any discernible differences in those binding sites, remains to be determined.

Summary and outlook of section

Few protein domains that bind α-1,3-glucan have been characterized in detail. In **Article II** we characterize the founding member of a new CBM family having a β-trefoil fold with such binding ability. β-trefoils are especially interesting as binding domains because of their symmetry that often facilitates multivalent binding, offering the potential of three binding sites. Despite being broadly recognised as lectins, only three CBM families with the β-trefoil fold have previously been reported. However, there are likely many more protein domains that could form new CBM families, either from unstudied sequences or from sequences that have previously been characterized as lectins. While the β-sheets are generally conserved in β-trefoils because of their importance to the structure, the connecting loops and turns offer a high variability in terms of sequence length and composition, providing functional diversity (102), which indeed can be seen across these four β-trefoil CBM families.

It is interesting to note that all described CBM domains connected to GH87 catalytic domains seem to belong to CBM families that have high variability in binding specificity, as for the β-sandwich domains from CBM6, CBM32 and CBM35, and, presumably, for CBMXXX (98, 102). This suggests that small sequence differences can alter the binding specificity in different protein folds and shows how difficult it can be to predict a CBM domain's binding ability solely based on the placement in a certain family. However, the binding ability of a CBM domain does not necessarily correspond to the activity of the catalytic domain but could also target an associated glycan that may be present in the same context, for example other parts of the fungal cell walls in the case of α-1,3-glucan. Thus, while a hint towards the binding specificity can often be given from the connected catalytic domain, it is important that such binding is verified.

4.2 Exploring nature

Since α-1,3-glucans do not accumulate in nature, we can assume that somehow these polysaccharides are being degraded. Microorganisms likely play the major role in this degradation, by using a variety of appropriate enzymes. Since not much is known about α-1,3-glucan degradation, with only a limited number of characterized enzymes from a narrow species diversity, it is likely that there are unknown enzymes with α-1,3-glucanase activity remaining to be found. For example, *Pseudomonas* species have been reported to produce α-1,3-glucanase but the activity have not been linked to a specific protein (66, 67). A strategy to find novel enzymes is therefore to search for microorganisms that can metabolize and thus break down the target polysaccharides. Here, I present my work on first searching for and identifying such organisms and then investigating their secretomes during growth on α-1,3-glucans to elucidate degradative enzymes.

4.2.1 Utilizing microorganism metabolism

The prevalence of α-1,3-glucan in nature

The two major known sources of α-1,3-glucan are fungal cell walls and biofilms (106), and we would therefore expect there to be microorganisms degrading α-1,3-glucan at least in natural environments where fungi live and are degraded. Microorganisms degrading α-1,3-glucan could also be present in bacterial biofilms, such as dental plaque. Indeed, a part of the life cycle of biofilm-residing organisms is active dispersion of the biofilm leading to colonization of other areas. This dispersion is often mediated in part by enzymes targeting the biofilm matrix polymers, produced either by the polymer-producing bacteria themselves or by other species residing in the biofilm (107). *S. mutans* has been shown to release cells from the biofilm through the production of the so-called surface-protein-releasing enzyme, a protease which targets a salivary receptor involved in adhesion of the biofilm to the tooth. *S. mutans* also produces dextranase during biofilm development, but this enzyme is believed to rather be involved in glucan remodelling or nutrient reacquisition (108).

There are thus several interesting environments that could be explored for the isolation of α-1,3-degrading organisms. In previous studies, successful isolation of such organisms has been achieved from soil and fermented foods (another source of α-glucan-containing biofilms) (109, 110). In my studies, I aimed to employ two strategies: the isolation of α-1,3-degrading organisms from soil and the characterization of an oral bacterium, *Prevotella scopos*, which encodes a putative polysaccharide utilization locus (111) that includes a GH87 enzyme. However, after some initial characterization and trials, the latter project was put on hold in favour of other projects and thus only the first project is mentioned in this thesis.

Isolation of α-1,3-glucan-degrading organisms from soil – results related to Article III

A convenient characteristic of insoluble polysaccharides such as α-1,3-glucan and alternan is that while the polysaccharides are insoluble, the degradation products, i.e. oligosaccharides and monosaccharides, are soluble. By incorporating the insoluble polysaccharides into agar (or agarose), one can produce semi-opaque cultivation plates. If the microorganisms grown on the plate can break down the insoluble polysaccharide embedded within the agar, a clearing zone around the microbial colony should appear (Fig. 4.10). If, however, the microorganism rather utilizes something else in the plate as the carbon source, such as the agar, then there might be visible growth but no clearing zone.

Figure 4.10. Microbial growth on an agar plate containing α-1,3-glucan. Around the colonies a clearing zone can be seen, indicating degradation of the insoluble (white) polysaccharide.

This strategy was chosen to try to find α-1,3-glucan-degrading microorganisms. Because α-1,3-glucan is present in many fungal cell walls, I collected samples from parks in Gothenburg at locations where fungi might have been growing or partially degraded. The selected samples were soil with woody residues adjacent to a fallen tree with a polypore fungus (Fig. 4.11, A), the mossy soil beneath a maple tree (B) and the leafy soil under a beech tree including an unidentified purple mushroom (C).

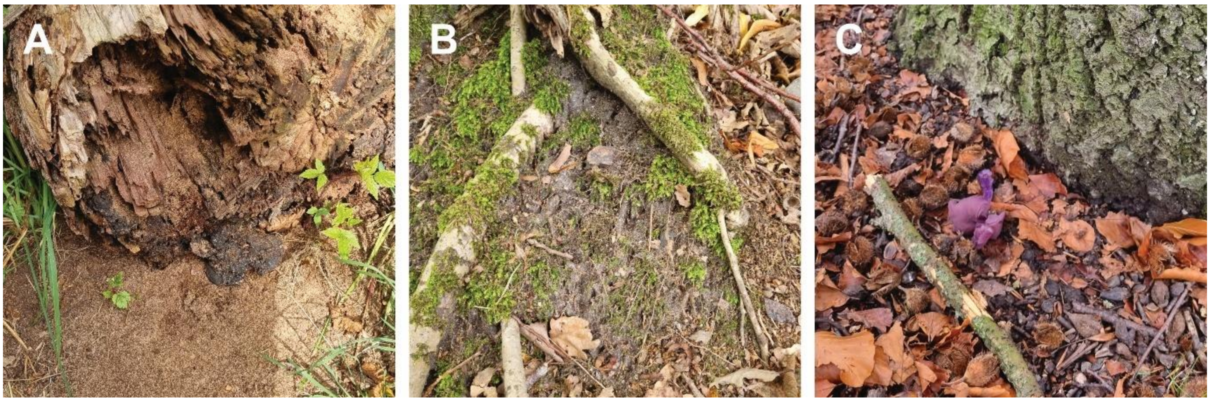


Figure 4.11. Sampling spots for the soil samples used for screening of α-1,3-glucan-degrading organisms. The isolate Mut1 originated from sawdust-like soil collected by a dead tree **(A)**, Mut2 from the mossy soil beneath a maple tree **(B)**, and Alt1, Alt2, Alt3 and Alt4 were all isolated from the leafy soil with an unidentified mushroom beneath a beech tree (C).

The collected samples were suspended in water and plated on α-1,3-glucan- or alternan-containing agar plates. Through repeated streaks on separate plates, and visual verification of their glucan-degrading ability, several pure isolates were acquired. Of the isolates, twelve from α-1,3-glucan plates and four from the alternan plates were subjected to colony PCR of the 16S rRNA region, and the products were sequenced for taxonomic identification. Of the α-1,3-glucan-isolates, six were *Paenibacillus*, two *Streptomyces* and for the rest the sequencing failed. Of the isolates from alternan, all four were *Streptomyces.* For further characterization, I chose the six *Streptomyces* isolates (Fig. 4.12) that were fully genome-sequenced. This was in part due to the relatively many α-1,3-glucanases already characterized from *Paenibacillus,* and because we were interested in the potential similarities or differences that could be found within the same genus from the two different isolation conditions. We gave the isolates names after their isolation polysaccharide, Mut1 and Mut2 for isolation on α-1,3-glucan (also known as mutan), and Alt1-4 for isolation on alternan.

Figure 4.12. The isolated and sequenced *Streptomyces* strains, growing on LB agar. Clear differences can be seen in the morphology of the strains, and although Alt1, Alt2 and Alt4 are the same species, Alt1 has different pigmentation.

Based on their genome sequences, we performed phylogenetic analyses resulting in the categorization of the isolates to species level, which was complemented by physiological experiments. Mut1 and Alt3 turned out to be previously undescribed species, and we named them *Streptomyces castrisilvae* and *Streptomyces glycanivorans,* Mut2 was a strain of *Streptomyces laculatispora* and Alt1, Alt2 and Alt4 were strains of *Streptomyces poriferorum*. The name *S. castrisilvae*, meaning "of castle forest" refers to the name of the park from which the species was isolated, Slottsskogen (SWE: castle forest). *S. glycanivorans*, meaning glycan devouring, was named based on the ability of this species to utilize a wide variety of glycans as carbon source.

A deeper analysis of the cazymes encoded by the organisms revealed that all isolates encoded a plethora of copies from multiple families, potentially enabling utilization of various polysaccharides from plant-, animal- and fungal biomasses (see Table 4, **Article III**). Their carbon source utilization was also tested experimentally based on this information, and growth was verified on many of the tested saccharides (Fig. 4.13 and Fig. 4, **Article III**).

If the selection criteria of being able to utilize α-1,3-glucan or alternan is indeed related to the organisms' ability of degrading fungal cell walls, they might be able to degrade chitin and β-glucan apart from α-1,3-glucan, since these polysaccharides are common constituents of fungal cell walls as well. Indeed, all isolates encode many enzymes in CAZy families with such activities. Of the experimentally tested growth substrates, all isolates except Mut1 could grow on yeast β-glucan, which consists of β-1,3-glucan with β-1,6 branches. Only Alt3 showed clear degradation of crustacean α-chitin, though structural differences in this type of chitin compared to that of fungal cell walls may not make the inability to grow indicative of the capability to growth on fungal chitin (112). One could speculate that the higher salt requirement of Alt3 indicates a native environment in or around the sea where the utilization of chitin from crustaceans is advantageous. In contrast to the growth experiments, all isolates but Alt3 produced *N*-acetyl-β-glucosaminidase, according to the API ZYM kit, indicating that these strains may have some chitin- or chitooligosaccharide-related degradative ability.

All the isolated species degraded α-1,3-glucan, but only the ones isolated on alternan could grow on that polysaccharide as a carbon source. Alt1-4 could also utilize dextran, indicating their capability for degrading polysaccharides with α-1,6-linkages. All isolates encoded GH87 enzymes: Mut1 encoded 6 copies, Mut2 encoded 2, and Alt1-4 encoded 4 copies each. Alt1-4, but not Mut1 and Mut2, also encoded an enzyme from GH49, which is a family known to contain dextranase activity (113). Potentially this enzyme could be responsible for the alternan- and dextran-degrading ability of the strains.

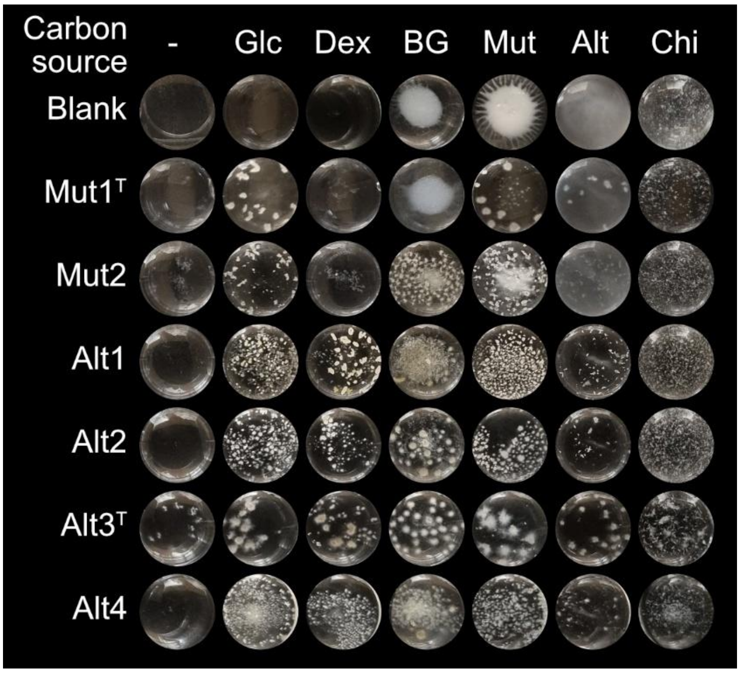


Figure 4.13. Growth of the isolated strains on various carbon sources. The growth on glucose (Glc), dextran (Dex), yeast β-glucan (BG), α-1,3-glucan (Mut), alternan (Alt), and shrimp shell chitin (Chi) are compared to the negative control (-), consisting of minimal media without carbon source but with the respective strain added, and the blank, in which the carbon source but not bacteria are added. Adapted from Fig. 4 in **Article III**, which displays additional tested carbon sources (114).

Summary and outlook of section

Streptomyces are mycelial bacteria with large genomes often encoding a large repertoire of cazymes (115). They are generally abundant in soil and are known to be involved in many degradative processes in nature, including the decomposition of lignocellulosic and fungal biomasses (116). Two GH87 enzymes, one α-1,3-glucanase and one mycodextranase, have previously been characterized from *Streptomyces* (85, 88). It is therefore not entirely surprising that we found members of this genus in our study. However, the metabolism of α-glucans have not been studied in *Streptomyces,* and while dextranases have been reported (117), the mechanisms that facilitate degradation of alternan remain unknown. It is also of interest to note the difference in degradative capabilities of the stains, especially relating to alternan and dextran, but also the inability of Mut1 to degrade β-glucan while at the same time being the best α-1,3-glucan degrader. If the target of these enzymes is indeed fungal cell walls, the lack of β-glucanase activity is surprising, perhaps suggesting a very specific niche of cell walls the organism can degrade. Overall, the study of polysaccharide utilization in *Streptomyces* is interesting both in terms of foundational understanding of these multifaceted microorganisms but also for the potential identification of useful enzymes, which is further explored in the next section.

4.2.2 Enzyme discovery from isolated microorganisms

Having isolated both α-1,3-glucan- and alternan-degrading species in **Article III**, we wanted to investigate what enzymes the organisms use to degrade these polysaccharides. A powerful method to identify proteins produced by an organism under certain conditions is proteomics, and with regards to polysaccharide-degrading cazymes, the secretome is of particular interest (118). This is especially true for insoluble substrates, where degradation takes place outside of the cell, but also for soluble, or semi-soluble substrates, where initial degradation often occurs outside the cell. For soluble substrates of interest, organisms can be grown in liquid cultures and the secretome simply collected by separation from the cells by centrifugation at the point of sample collection. However, for explorations of insoluble polysaccharide degradation, such conventional methods have some issues. If cells are grown in liquid cultures with suspended insoluble material and then removed by centrifugation, the polysaccharides would also end up in the pellet. If there are enzymes with high affinity for the polysaccharide, potentially mediated by the presence of CBM domains, these proteins may be bound to the polysaccharide and removed during sample preparation. Trying to separate such proteins from the substrate, through for example boiling or adding surfactants, one instead risks damaging the cells and co-extracting contaminating intracellular proteins (119). To get around such issues, Bengtsson et al. developed a secretomics sample preparation method involving cultivation on agar or agarose plates incorporating the carbon source of choice (119), which was later shown to indeed contain a higher fraction of truly secreted proteins than in corresponding samples from liquid cultures (120). The method involves growth of the organism on a double layered plate with a membrane placed in the middle of the setting agent. The organism is grown and retained to the top of the plate whereas secreted enzymes can permeate through the membrane and therefore be separated and collected by flipping the plate upside down to extract samples from underneath the membrane (Fig. 4.14).

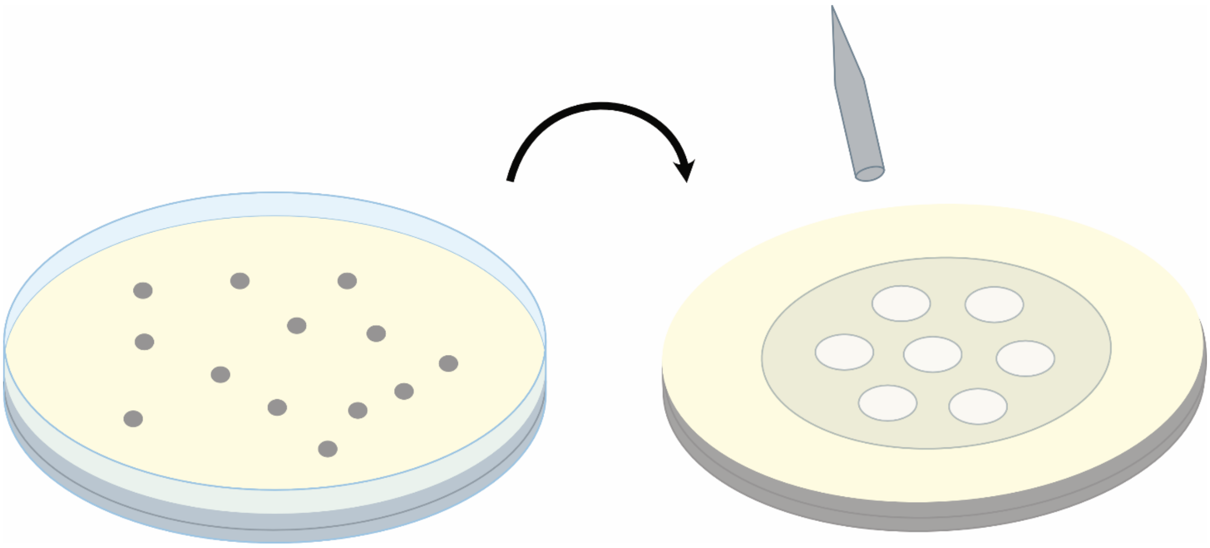
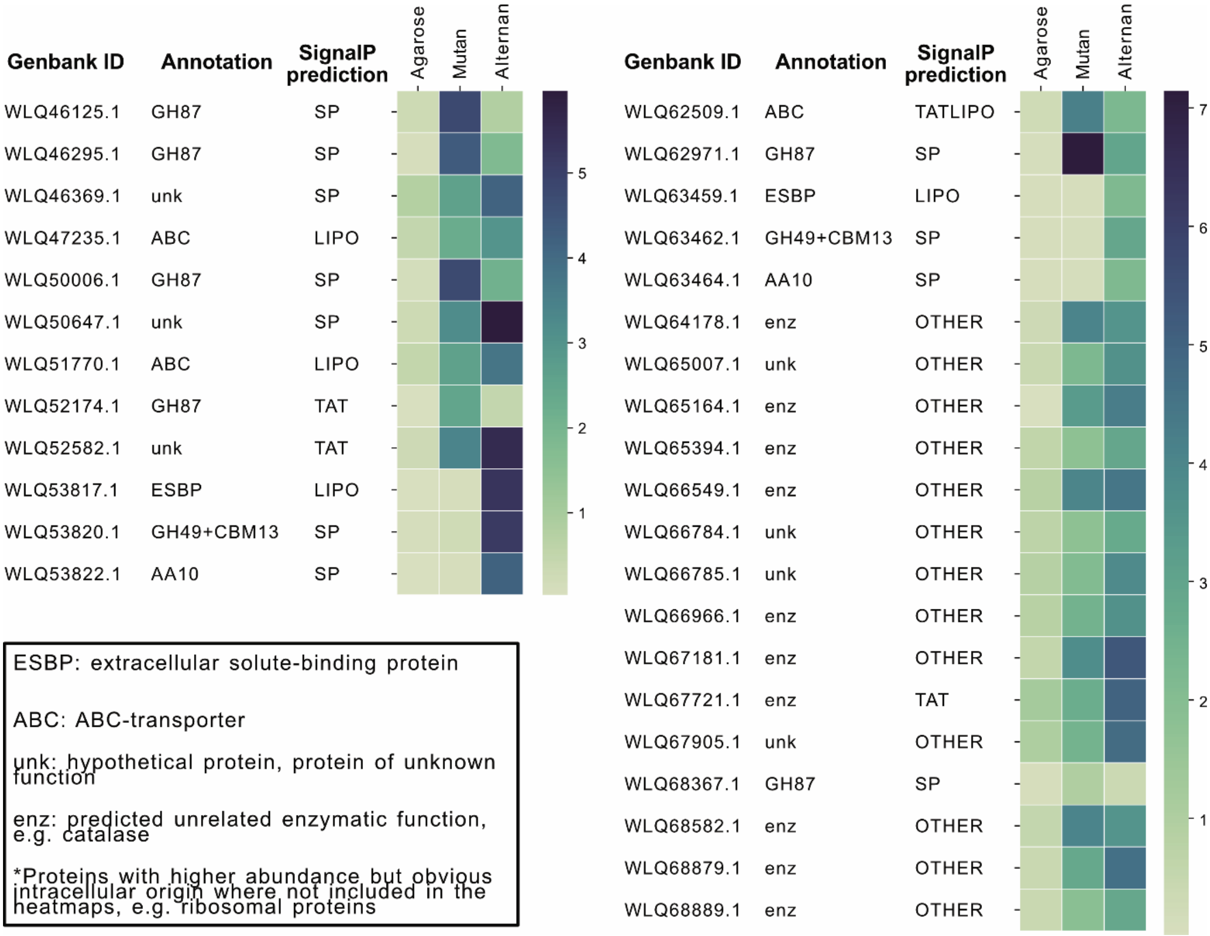


Figure 4.14. Schematic of the secretomics plate method. A double layered agarose plate is poured with a membrane between the layers. The microorganism is plated on top and after growth, the plate can be flipped and cell free samples collected from underneath the membrane.

Results related to Article IV

In **Article IV** we used the above-mentioned plate method to study the secretome of two of the strains isolated in **Article III**, *S. poriferorum* Alt1 and *S. glycanivorans* Alt3. I explored their secretomes following growth on α-1,3-glucan (mutan) or alternan compared to their growth on agarose as a control. Several proteins were detected in higher abundance in either or both of these conditions compared to the control. Furthermore, several of the detected proteins were annotated as cazymes (Fig. 4.15). The samples from *S. glycanivorans* contained more proteins in general, including proteins in higher abundance in the conditions of interest. However, many of the detected proteins were predicted to be intracellular, indicating that some degree of cell lysis had occurred. The detected proteins were quite similar in the two species, and the most interesting discoveries were the GH87 enzymes detected in both conditions, but primarily in the α-1,3-glucan-derived samples, and three proteins located in a putative cluster comprising an extracellular solute-binding protein, a GH49-CBM13 enzyme, and a AA10 enzyme that was detected only in the alternan samples.



Genbank ID	Annotation	SignalP prediction
WLQ46125.1	GH87	SP
WLQ46295.1	GH87	SP
WLQ46369.1	unk	SP
WLQ47235.1	ABC	LIPO
WLQ50006.1	GH87	SP
WLQ50647.1	unk	SP
WLQ51770.1	ABC	LIPO
WLQ52174.1	GH87	TAT
WLQ52582.1	unk	TAT
WLQ53817.1	ESBP	LIPO
WLQ53820.1	GH49+CBM13	SP
WLQ53822.1	AA10	SP

Genbank ID	Annotation	SignalP prediction
WLQ62509.1	ABC	TATLIPO
WLQ62971.1	GH87	SP
WLQ63459.1	ESBP	LIPO
WLQ63462.1	GH49+CBM13	SP
WLQ63464.1	AA10	SP
WLQ64178.1	enz	OTHER
WLQ65007.1	unk	OTHER
WLQ65164.1	enz	OTHER
WLQ65394.1	enz	OTHER
WLQ66549.1	enz	OTHER
WLQ66784.1	unk	OTHER
WLQ66785.1	unk	OTHER
WLQ66966.1	enz	OTHER
WLQ67181.1	enz	OTHER
WLQ67721.1	enz	TAT
WLQ67905.1	unk	OTHER
WLQ68367.1	GH87	SP
WLQ68582.1	enz	OTHER
WLQ68879.1	enz	OTHER
WLQ68889.1	enz	OTHER

ESBP: extracellular solute-binding protein

ABC: ABC-transporter

unk: hypothetical protein, protein of unknown function

enz: predicted unrelated enzymatic function, e.g. catalase

*Proteins with higher abundance but obvious intracellular origin where not included in the heatmaps, e.g. ribosomal proteins

Figure 4.15. Heat maps of protein abundance of proteins that were of significantly higher abundance in at least one condition compared to the control. From Fig. 1 in **Article IV**.

Detected GH87 proteins

Both species encode four copies of GH87 enzymes, denoted by respective species indicator, *Sp* or *Sg*, and a letter based on the order of their locus tags. Compared to the control, all of these were found in higher abundance in the α-1,3-glucan growth conditions for *S. poriferorum* and two of them for *S. glycanivorans.* In the alternan conditions the difference in abundance to the control was less pronounced but significantly higher for *Sp*GH87B, *Sp*GH87D, *Sg*GH87A and *Sg*GH87D. Interestingly, the four copies of GH87 enzymes have different domain architectures, with varying numbers of appended CBM32 domains (Fig. 4.16). While CBM32 domains have not been demonstrated to bind α-1,3-glucan before, such domains are often present together with GH87 enzymes (90) and they are somewhat similar (33-60 % sequence identity) to the DS1 and DS2 domains connected to the GH87 enzyme Agl-KA, which were shown to bind α-1,3-glucan by Suyotha et al (91) (mentioned in section 4.1.4). The catalytic GH87 domains of the proteins also differ in similarity with between 29-90 % sequence identity to each other. Compared to other characterized GH87 enzymes, the closest related proteins for most of the proteins in our study is to other *Streptomyces* proteins. *Sp*GH87A is most similar to the mycodextranase Myd (88) and *Sp*GH87C, *Sp*GH87D and *Sg*GH87A are most similar to the α-1,3-glucanase AglsT from *Streptomyces thermodiastaticus* HF 3-3(85). The similarities between these proteins are relatively low (37-44 %) while, curiously, the similarity between *Sp*GH87B and *Sg*GH87D to MuC1 from *Paenibacillus* sp. KSM-M126 (83) is much higher (~76 %), perhaps indicating different evolutionary origin of these proteins. The fact that the closest related characterized enzymes are ones with different activity also indicates varying biological functions for the proteins detected in our study, and provides a likely rationale for the multiple copies of GH87 enzyme encoded by *S. poriferorum* and *S. glycanivorans.*

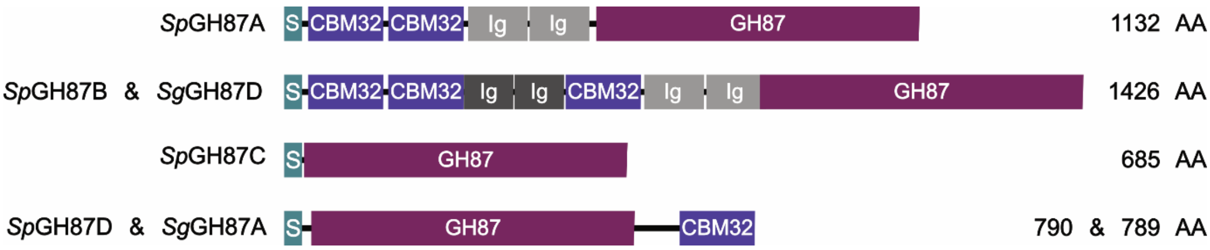


Figure 4.16. Domain architecture of GH87 enzymes detected in *S. poriferorum* and *S. glycanivorans.* S – signal peptide, Ig – immunoglobulin-like, CBM32 – carbohydrate-binding family 32 module. Different shading of the Ig-like domains indicates the two different types found in the proteins.

Discovery of an alternan-associated gene cluster

Three proteins, with homologs in both species, were found in higher abundance in the alternan samples compared to both α-1,3-glucan and the agarose control. These were extracellular solute binding proteins, multimodular GH49-CBM13 proteins and AA10 enzymes. The genes encoding these proteins were also near each other in the genome, suggesting the possibility of a co-regulated gene cluster. In addition to the proteins detected in the proteomics experiment, the putative clusters also contained ABC-transporter proteins, GH13 enzymes, ROK-family proteins and DoxX-family proteins (Fig. 4.17).

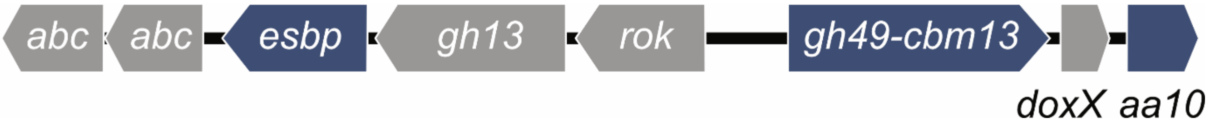


Figure 4.17. Putative gene cluster found in both *S. poriferorum* and *S. glycanivorans*. The encoded proteins that were upregulated during growth on alternan in the proteomics experiment are marked in blue. The other proteins all lack predicted signal peptides and are predicted to have related functions.

The combination of these proteins seems likely to constitute Gram-positive polysaccharide utilization loci (gpPULs) which in general comprise transcriptional regulators, transporters and multiple cazymes (121). The ROK protein family consist of transcriptional repressors, uncharacterized open reading frames and sugar kinases, making the ROK-protein a likely regulator of the respective locus (122). ESBPs are ligand-binding proteins that deliver molecules to ABC-transporters for transport across the cell membrane (123). To investigate the clusters' function, we studied the ESBP, GH13, GH49, CBM13 and AA10 proteins in more detail via heterologous production in *E. coli*. The GH49 enzymes had endo-like activity on alternan and dextran polysaccharides, as well as on isomaltooligosaccharides with higher degree of polymerization than five. The degradation products were a range of oligosaccharides, but with isomaltotriose as the minimal length. The GH13 enzymes were exo-acting α-1,6-glucosidases degrading the resulting oligosaccharides to glucose but with no discernible activity on the polysaccharides. The ESBPs showed binding to isomaltose and even higher affinity to isomaltotriose, indicating their ability to facilitate transport between the extracellular GH49 enzymes to the intracellular GH13 glucosidases. The CBM13 domain appended to the *Sp*GH49 domain was shown to bind alternan, further establishing it as a substrate.

The function of the AA10 enzymes in the clusters remains unknown. Other characterized AA10 enzymes have oxidative activity on chitin or cellulose, and while both oxidase and peroxidase activities were confirmed, verifying the proteins as active LPMO enzymes, their addition to enzyme reactions did not result in any detectable difference on any of the tested substrates. An interesting difference between *Sp*LPMO10F and *Sg*LPMO10C to other AA10 enzymes are some key amino acid residues (Fig. S9, **Article IV**) that have been shown to be involved in substrate specificity (124, 125). These create distinct binding surfaces in *Sp*LPMO10F and *Sg*LPMO10C, suggesting that the target substrate might be different from previously characterized enzymes, but the identity of the putative substrate remains to be determined. One possibility is that the lyophilized alternan used in the experiments has an altered crystallinity compared to the natural substrate, as is the case with α-1,3-glucan (126), making the enzymes unable to interact with it in a productive manner. Another hypothesis is that the enzymes are not involved in polysaccharide degradation but instead are involved in redox stress mitigation or another activity where they utilize hydrogen peroxide.

Summary and outlook of section

With the exception of amylases degrading starch, enzymes facilitating α-glucan utilisation in microorganisms have not been extensively studied. Two studies have characterized the dextran-targeting polysaccharide utilization locus from *F. johnsoniae* (127, 128). Though fundamentally different to the gpPULs from *S. poriferorum* and *S. glycanivorans* in terms of included enzymes, an interesting parallel is that this PUL seems to specifically target dextran with α-1,2-branches. Though we cannot say that the gpPULs in our study specifically targets alternan, it is recognised as a substrate and the GH49 enzymes seem to be able to accommodate some degree of α-1,3-linkages in their active sites and the CBM13 domains are able to bind it. CBM13 domains have not previously been associated with α-1,3- or α-1,6-glucans, with the exception of the domain found appended to the GH27 dextranase characterized in the aforementioned PUL (128). However, that domain was not characterized itself, nor together with the catalytic domain, so its binding ability remains unstudied. The binding abilities of CBM13 domains are diverse (129), and whether the affinity for alternan is recurring is a topic for the future. Another mystery is the activity of the AA10 enzymes. If they are active on a carbohydrate, and this specificity could be elucidated, perhaps this could give an indication of the natural target of this type of gpPUL.

These two species most likely cleave α-1,3-glucan and the α-1,3-linkages in alternan using one or more of the GH87 enzymes. The varying domain architectures and sequences of the catalytic domains indicate some variations in the activity of these enzymes. It may be that the similarity of *Sp*GH87A to mycodextranase is an indication that it prefers or can accommodate other linkages than α-1,3, enabling the degradation of alternan. Though the interplay between the GH87 enzymes and the proteins in the gpPUL is not fully resolved, the activities of the enzymes allow us to construct a model to illustrate the α-glucan utilization in *S. poriferorum* and *S. glycanivorans*. Here, the polysaccharides are cleaved by GH87, GH49 and potentially AA10 enzymes. The ESBP can bind to resulting isomaltooligosaccharides and facilitate transport through the ABC-transporter. Inside the cell, the GH13 enzymes can cleave isomaltooligosaccharides into glucose that would go into the central metabolism (Fig 4.18).

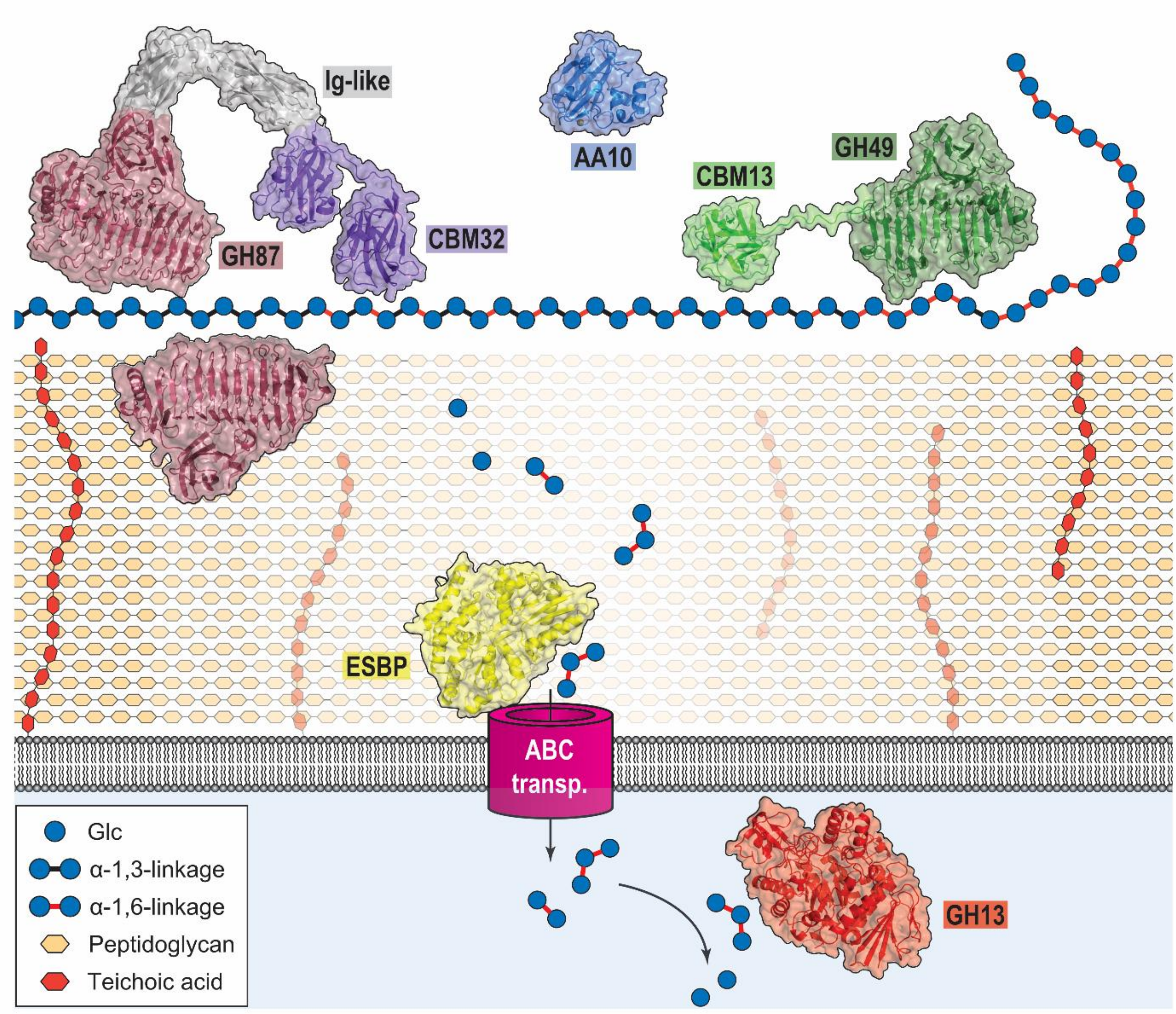


Figure 4.18. Model of α-glucan utilization in *S. poriferorum* and *S. glycanivorans*. Secreted enzymes cleave the polysaccharides, with GH87 enzymes acting on α-1,3-linkages and GH49 enzymes on α-1,6-linkages. The CBMs appended to the catalytic domains facilitate polysaccharide binding. The AA10 enzymes presumably either disrupt crystalline structures in the polysaccharides or mitigate redox stress. The ESBPs bind to oligosaccharides and shuttle them to the ABC-transporter for import into the cell where GH13 enzymes degrade α-1,6-linked oligosaccharides into glucose.

Chapter 5: Conclusions

In this thesis I have described my work aiming to expand the knowledge of enzymes and proteins targeting α-1,3-glucan. Divided into two main strategies, my work encompasses the exploration of the known CAZy families where α-1,3-glucanase activity have been found, GH71 and GH87, and an activity-driven exploration of the α-1,3-glucan- and alternan-degrading capacity of microorganisms.

In **Article I**, we presented the first crystal structure with ligands of an enzyme from GH71 and coupled it with biochemical studies to identify the catalytic amino acids and the mechanism of the enzymes. Our work contributed with fundamental structural and mechanistic insight into a previously understudied enzyme family, which provide a foundation for future functional studies and engineering efforts.

In **Article II**, a multimodular enzyme with a GH87 catalytic domain was characterized. This work led to the identification of a previously unrecognized CBM domain with affinity for both α-1,3-glucan and alternan. Detailed characterization of this CBM domain revealed two distinct binding sites and identified the amino acid residues involved in ligand interaction. These results provide valuable information of this newly discovered CBM family, establishing a framework for future studies of such domains. Specifically, the study details the characteristics of the founding member and facilitate its utilization in targeting α-1,3-glucan.

Article III describes the activity-based screening of soil samples leading to the discovery of several new *Streptomyces* strains and species capable of metabolising α-1,3-glucan and/or alternan. Characterization of these isolates demonstrated that they are highly efficient glycan degraders, encoding a diverse repertoire of cazymes and exhibiting the ability to grow on a wide range of carbohydrates and can therefore serve as potential sources for discovery of novel enzymes. I think that the strategy of specifically searching for α-1,3-glucan- and alternan-degrading organisms was highly successful, and believe that a similar approach would be applicable also for other understudied polysaccharides, for example from other biofilm types.

Building on these findings, **Article IV** investigated the secretomes of the two isolates *S. poriferorum* Alt1 and *S. glycanivorans* Alt3 during growth of α-1,3-glucan and alternan. This analysis identified multiple GH87 enzymes being abundant during growth on these polysaccharides, as well as a set of proteins abundant only during growth on alternan. The genes encoding these proteins were located in a cluster which collectively encoded proteins with complementary activities, including the degradation alternan and dextran, oligosaccharide import and intracellular hydrolysis into glucose. Description of such a utilization system is the first of its kind in Gram-positive bacteria and provides knowledge how alternan and dextran can be broken down.

Collectively, the work presented in this thesis advances the understanding of α-1,3-glucanases and α-1,3-glucan-binding proteins, fulfilling the overall aim of the thesis.

5.1 Future perspectives

Progress in the characterization of α-1,3-glucanases has been relatively slow since their initial discovery. A contributing factor is the lack of commercially available and well-defined α-1,3-glucan substrates, as the substrates used often originate from different sources, are prepared using diverse methodologies, and may vary in composition. As a result, it is frequently difficult to directly compare experimental outcomes across studies and, in some cases, even to draw clear conclusions regarding enzyme specificity and mode of action. Despite this limited fundamental understanding, the biological relevance of α-1,3-glucans has driven early application-focused studies of α-1,3-glucanases, highlighting their potential in targeting both fungal cell walls and dental plaque. Plants expressing α-1,3-glucanases have shown increased resistance against fungal pathogens (130, 131) and α-1,3-glucanases have been investigated as additives in oral healthcare products such as mouthwashes and chewing gums (132, 133).

Similarly to foundational studies, investigations of enzyme performance in complex systems, such as biofilms and fungal cell walls, are also hampered by difficulties in making meaningful comparisons and conclusions. For example, *in vitro* studies of α-1,3-glucanases targeting biofilms often differ in experimental setups including the biofilm model itself (what or which organisms are used), the enzymes used (which and at what concentration), or other conditions affecting biofilm formation and enzyme performance (pH, buffering, addition of sucrose, experiment timescale, etc.) (134). These limitations underscore the need for broader comparative studies employing diverse enzymes, both within and between GH71 and GH87, under standardized conditions or in the same study. Conversely, the use of the same enzymes on substrates from different sources is also of relevance. Such studies could help identify highly active enzymes and determine whether particular enzyme types are better suited for specific applications.

Although mechanisms are now elucidated and structural data exist for both GH71 and GH87, they are still very much understudied with much of their diversity unexplored. In GH71, diverse members of the fungal portion of the phylogenetic tree have been characterized but the function of the bacterial enzymes in the family remains completely unknown (Fig. 4.1). In GH87, only enzymes from a limited number of genera have been studied to date. Of interest is also the sole characterized mycodextranase in the family (88). Since this enzyme cleaves α-1,4-linkages of nigeran (alternating α-1,3- and α-1,4-glucan), while all other characterized members of the family are α-1,3-glucanases, it indicates an underexplored functional diversity of activities within GH87. Identifying and characterizing additional enzymes across the phylogenetic tree could provide insight into the amino acid determinants governing substrate specificity. A strategy for exploration of diversity could be to explore enzymes from organisms encoding multiple copies of GH87 enzymes, such as the enzymes detected to be secreted by *S. poriferorum* and *S. glycanivorans* in **Article IV**. It is likely that at least some of these enzymes fulfil distinct biological roles, but whether this is mediated by differences in the catalytic domains or by the different appended types of CBMs is an open question.

CBMs binding α-1,3-glucan have been even less studied than catalytic domains cleaving the same polysaccharide. Domains from the GH87 enzyme Agl-KA have been fused to fluorescent proteins and utilized for labelling of fungal cell wall and for induced aggregation α-1,3-glucan, inhibiting fungal biofilm formation (135). However, the individual binding domains used in these studies only bound weakly to α-1,3-glucan, and stronger binding therefore required the presence of all three domains. It would be interesting to study how well *Fj*CBMXXX$_{\text{GH87}}$ compares to these and other binding domains, and if the multivalency could promote higher affinity. Additionally, the use of α-1,3-glucan-binding CBMs in conjunction with α-1,3-glucanases could be explored as a strategy to improve enzyme retention and efficacy in fluctuating environments such as the oral cavity. Beyond α-1,3-glucan binding, the newly described CBMXXX family is of broader interest. The presence of CBMXXX domains appended to diverse catalytic domains suggests that the family may encompass a wider range of binding specificities than to just α-1,3-glucan. The multivalent nature of these domains, with potential for up to three binding sites, makes them especially attractive candidates for protein engineering and biotechnological applications.

In conclusion, the knowledge on proteins targeting α-1,3-glucan is still in its infancy, but new foundational understanding, as partly provided in this thesis, now provides an opportunity for further research and development. By having a better understanding of enzyme specificity, catalytic mode of action, and insights into the significance of protein domain architectures, more rational experiments can be conducted and conclusions drawn, which can lead to optimized applications for a real-life context.

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References

1. Davies, G. J., and Sinnott, M. L. (2008) Sorting the diverse: The sequence-based classifications of carbohydrate-active enzymes. *The Biochemist*. **30**, 26–32
2. Varki, A. (2017) Biological roles of glycans. *Glycobiology*. **27**, 3–49
3. Yoshimi, A., Miyazawa, K., and Abe, K. (2017) Function and biosynthesis of cell wall α-1,3-glucan in fungi. *J. Fungi*. **3**, 1–21
4. Koo, H., Falsetta, M. L., and Klein, M. I. (2013) The exopolysaccharide matrix: a virulence determinant of cariogenic biofilm. *J. Dent. Res.* **92**, 1065–1073
5. Chapple, I. L. C., Van Der Weijden, F., Doerfer, C., Herrera, D., Shapira, L., Polak, D., Madianos, P., Louropoulou, A., Machtei, E., Donos, N., Greenwell, H., Van Winkelhoff, A. J., Eren Kuru, B., Arweiler, N., Teughels, W., Aimetti, M., Molina, A., Montero, E., and Graziani, F. (2015) Primary prevention of periodontitis: Managing gingivitis. *J. Clin. Periodontol.* **42**, S71–S76
6. Wade, W. G. (2013) The oral microbiome in health and disease. *Pharmacol. Res.* **69**, 137–143
7. World Health Organization (2022) *Global oral health status report: towards universal health coverage for oral health by 2030*, World Health Organization
8. Jakubovics, N. S., Goodman, S. D., Mashburn-Warren, L., Stafford, G. P., and Cieplik, F. (2021) The dental plaque biofilm matrix. *Periodontol. 2000*. 10.1111/prd.12361
9. Valm, A. M. (2019) The structure of dental Plaque microbial communities in the transition from health to dental caries and periodontal disease. *J. Mol. Biol.* **431**, 2957–2969
10. Marsh, P. D. (2004) Dental plaque as a microbial biofilm. *Caries Res.* **38**, 204–211
11. Baty, J. J., Stoner, S. N., and Scoffield, A. (2022) Oral commensal streptococci: Gatekeepers of the oral cavity
12. Kilian, M., Chapple, I. L. C., Hannig, M., Marsh, P. D., Meuric, V., Pedersen, A. M. L., Tonetti, M. S., Wade, W. G., and Zaura, E. (2016) The oral microbiome - An update for oral healthcare professionals. *Br. Dent. J.* **221**, 657–666
13. Rostami, N., Shields, R. C., Yassin, S. A., Hawkins, A. R., Bowen, L., Luo, T. L., Rickard, A. H., Holliday, R., Preshaw, P. M., and Jakubovics, N. S. (2017) A Critical Role for Extracellular DNA in Dental Plaque Formation. *J. Dent. Res.* **96**, 208–216
14. Crouch, E., and Wright, J. R. (2001) Glucan-binding proteins of the oral streptococci
15. Bowen, W. H., and Koo, H. (2011) Biology of *Streptococcus mutans*-derived glucosyltransferases: Role in extracellular matrix formation of cariogenic biofilms. *Caries Res.* **45**, 69–86
16. Rozen, R., Bachrach, G., Bronshteyn, M., Gedalia, I., and Steinberg, D. (2001) The role of fructans on dental biofilm formation by *Streptococcus sobrinus*, *Streptococcus mutans*, *Streptococcus gordonii* and *Actinomyces viscosus*. *FEMS Microbiol. Lett.* **195**, 205–210
17. Simpson, C. L., Cheetham, N. W. H., and Jacques, N. A. (1995) Four glucosyltransferases, GtfJ, GtfK, GtfL and GtfM, from *Streptococcus salivarius* ATCC 25975. *Microbiology*. **141**, 1451–1460
18. Ernst, L., Schulz, C., Petzold, A., Thurn-Albrecht, T., Saalwächter, K., and Wefers, D. (2024) Detailed structural characterization of five water-insoluble α-glucans produced by glucansucrases from *Streptococcus* spp. *Carbohydr. Polym.* **337**, 122164
19. Hoshino, T., and Fujiwara, T. (2022) The findings of glucosyltransferase enzymes derived from oral streptococci. *Jpn. Dent. Sci. Rev.* **58**, 328–335
20. Pleszczyńska, M., Wiater, A., Janczarek, M., and Szczodrak, J. (2015) (1→3)-α-d-Glucan hydrolases in dental biofilm prevention and control: A review. *Int. J. Biol. Macromol.* **79**, 761–778
21. Miyazaki, T. (2023) Glycoside hydrolases active on microbial exopolysaccharide α-glucans: structures and function. *Essays Biochem.* 10.1042/ebc20220219
22. Xiao, J., Klein, M. I., Falsetta, M. L., Lu, B., Delahunty, C. M., Yates, J. R., Heydorn, A., and Koo, H. (2012) The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. *PLoS Pathog.* **8**, 7–9
23. Ramakrishnan, R., Singh, A. K., Singh, S., Chakravortty, D., and Das, D. (2022) Enzymatic dispersion of biofilms: an emerging biocatalytic avenue to combat biofilm-mediated microbial infections. *J. Biol. Chem.* **298**, 102352
24. Gow, N. A. R., and Lenardon, M. D. (2023) Architecture of the dynamic fungal cell wall. *Nat. Rev. Microbiol.* **21**, 248–259
25. Gow, N. A. R., Latge, J.-P., and Munro, C. A. (2017) The fungal cell wall: structure, biosynthesis, and function. *Microbiol. Spectr.* **5**, 10.1128/microbiolspec.funk-0035–2016
26. Chakraborty, A., Fernando, L. D., Fang, W., Dickwella Widanage, M. C., Wei, P., Jin, C., Fontaine, T., Latgé, J. P., and Wang, T. (2021) A molecular vision of fungal cell wall organization by functional genomics and solid-state NMR. *Nat. Commun.* **12**, 1–12
27. Yao, R. A., Berrin, J.-G., McKee, L. S., and Bissaro, B. (2025) Fungal cell walls: the rising importance of carbohydrate-active enzymes. *Trends Microbiol.* **33**, 1085–1098

28. Kang, X., Kirui, A., Muszyński, A., Widanage, M. C. D., Chen, A., Azadi, P., Wang, P., Mentink-Vigier, F., and Wang, T. (2018) Molecular architecture of fungal cell walls revealed by solid-state NMR. *Nat. Commun.* **9**, 1–12
29. Zonneveld (1972) The significance of alpha-1,3-glucan of the cell wall and alpha-1,3-glucanasea for cleistothecium development. **273**, 174–187
30. Beauvais, A., Bozza, S., Kniemeyer, O., Formosa, C., Balloy, V., Henry, C., Roberson, R. W., Dague, E., Chignard, M., Brakhage, A. A., Romani, L., and Latgé, J.-P. (2013) Deletion of the α-(1,3)-glucan synthase genes induces a restructuring of the conidial cell wall responsible for the avirulence of *Aspergillus fumigatus*. *PLoS Pathog.* **9**, e1003716
31. Altered expression of surface alpha-1,3-glucan in genetically related strains of *Blastomyces dermatitidis* that differ in virulence 10.1128/iai.62.8.3543-3546.1994
32. Villalobos-Duno, H., San-Blas, G., Paulinkevicius, M., Sánchez-Martín, Y., and Nino-Vega, G. (2013) Biochemical characterization of *Paracoccidioides brasiliensis* α-1,3-glucanase Agn1p, and its functionality by heterologous expression in *Schizosaccharomyces pombe*. *PLoS ONE*. 10.1371/journal.pone.0066853
33. Rappleye, C. A., Eissenberg, L. G., and Goldman, W. E. (2007) *Histoplasma capsulatum* α-(1,3)-glucan blocks innate immune recognition by the β-glucan receptor. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 1366–1370
34. Fujikawa, T., Kuga, Y., Yano, S., Yoshimi, A., Tachiki, T., Abe, K., and Nishimura, M. (2009) Dynamics of cell wall components of *Magnaporthe grisea* during infectious structure development. *Mol. Microbiol.* **73**, 553–570
35. Buchanan, K. L., and Murphy, J. W. (1998) What makes *Cryptococcus neoformans* a pathogen? *Emerg. Infect. Dis.* **4**, 71–83
36. Reese, A. J., Yoneda, A., Breger, J. A., Beauvais, A., Liu, H., Griffith, C. L., Bose, I., Kim, M.-J., Skau, C., Yang, S., Sefko, J. A., Osumi, M., Latge, J.-P., Mylonakis, E., and Doering, T. L. (2007) Loss of cell wall alpha(1-3) glucan affects *Cryptococcus neoformans* from ultrastructure to virulence. *Mol. Microbiol.* **63**, 1385–1398
37. Laine, R. A. (1994) A calculation of all possible oligosaccharide isomers both branched and linear yields 1.05 × 1012 structures for a reducing hexasaccharide: the isomer barrier to development of single-method saccharide sequencing or synthesis systems. *Glycobiology*. **4**, 759–767
38. Varki, A., Cummings, R. D., Aebi, M., Packer, N. H., Seeberger, P. H., Esko, J. D., Stanley, P., Hart, G., Darvill, A., Kinoshita, T., Prestegard, J. J., Schnaar, R. L., Freeze, H. H., Marth, J. D., Bertozzi, C. R., Etzler, M. E., Frank, M., Vliegenthart, J. F., Lütteke, T., Perez, S., Bolton, E., Rudd, P., Paulson, J., Kanehisa, M., Toukach, P., Aoki-Kinoshita, K. F., Dell, A., Narimatsu, H., York, W., Taniguchi, N., and Kornfeld, S. (2015) Symbol nomenclature for graphical representations of glycans. *Glycobiology*. **25**, 1323–1324
39. Drula, E., Garron, M.-L., Dogan, S., Lombard, V., Henrissat, B., and Terrapon, N. (2022) The carbohydrate-active enzyme database: functions and literature. *Nucleic Acids Res.* **50**, D571–D577
40. Lapébie, P., Lombard, V., Drula, E., Terrapon, N., and Henrissat, B. (2019) Bacteroidetes use thousands of enzyme combinations to break down glycans. *Nat. Commun.* **10**, 2043
41. Lillington, S. P., Leggieri, P. A., Heom, K. A., and O'Malley, M. A. (2020) Nature's recyclers: anaerobic microbial communities drive crude biomass deconstruction. *Curr. Opin. Biotechnol.* **62**, 38–47
42. F. Wardman, J., and G. Withers, S. (2024) Carbohydrate-active enzyme (CAZyme) discovery and engineering via (Ultra)high-throughput screening. *RSC Chem. Biol.* **5**, 595–616
43. Thapa, S., Mishra, J., Arora, N., Mishra, P., Li, H., O′Hair, J., Bhatti, S., and Zhou, S. (2020) Microbial cellulolytic enzymes: diversity and biotechnology with reference to lignocellulosic biomass degradation. *Rev. Environ. Sci. Biotechnol.* **19**, 621–648
44. Wardman, J. F., Bains, R. K., Rahfeld, P., and Withers, S. G. (2022) Carbohydrate-active enzymes (CAZymes) in the gut microbiome. *Nat. Rev. Microbiol.* **20**, 542–556
45. McDonald, A. G., Boyce, S., and Tipton, K. F. (2009) ExplorEnz: the primary source of the IUBMB enzyme list. *Nucleic Acids Res.* **37**, D593–D597
46. McDonald, A. G., and Tipton, K. F. (2014) Fifty-five years of enzyme classification: advances and difficulties. *FEBS J.* **281**, 583–592
47. Henrissat, B., and Davies, G. (1997) Structural and sequence-based classification of glycoside hydrolases. *Curr. Opin. Struct. Biol.* **7**, 637–644
48. Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res.* **37**, D233–D238
49. Levasseur, A., Drula, E., Lombard, V., Coutinho, P. M., and Henrissat, B. (2013) Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. *Biotechnol. Biofuels*. **6**, 41
50. Davies, G., and Henrissat, B. (1995) Structures and mechanisms of glycosyl hydrolases. *Structure*. **3**, 853–859
51. Davies, G. J., Wilson, K. S., and Henrissat, B. (1997) Nomenclature for sugar-binding subsites in glycosyl hydrolases. *Biochem. J.* **321**, 557–559
52. McCarter, J. D., and Stephen Withers, G. (1994) Mechanisms of enzymatic glycoside hydrolysis. *Curr. Opin. Struct. Biol.* **4**, 885–892

53. Gloster, T. M., Turkenburg, J. P., Potts, J. R., Henrissat, B., and Davies, G. J. (2008) Divergence of catalytic mechanism within a glycosidase family provides insight into evolution of carbohydrate metabolism by human gut flora. *Chem. Biol.* **15**, 1058–1067
54. Vuong, T. V., and Wilson, D. B. (2010) Glycoside hydrolases: Catalytic base/nucleophile diversity. *Biotechnol. Bioeng.* **107**, 195–205
55. Bissaro, B., Monsan, P., Fauré, R., and O'Donohue, M. J. (2015) Glycosynthesis in a waterworld: new insight into the molecular basis of transglycosylation in retaining glycoside hydrolases. *Biochem. J.* **467**, 17–35
56. Molina, M., Cioci, G., Moulis, C., Séverac, E., and Remaud-Siméon, M. (2021) Bacterial α-glucan and branching sucrases from GH70 family: discovery, structure–function relationship studies and engineering. *Microorganisms*. **9**, 1607
57. Withers, S., and Williams, S. Glycoside hydrolases. *CAZypedia*. [online] http://www.cazypedia.org/index.php?title=Glycoside_hydrolases&oldid=19467 (Accessed January 7, 2026)
58. Hervé, C., Rogowski, A., Blake, A. W., Marcus, S. E., Gilbert, H. J., and Knox, J. P. (2010) Carbohydrate-binding modules promote the enzymatic deconstruction of intact plant cell walls by targeting and proximity effects. *Proc. Natl. Acad. Sci.* **107**, 15293–15298
59. Boraston, A. B., Bolam, D. N., Gilbert, H. J., and Davies, G. J. (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. *Biochem. J.* **382**, 769–781
60. Gilbert, H. J., Knox, J. P., and Boraston, A. B. (2013) Advances in understanding the molecular basis of plant cell wall polysaccharide recognition by carbohydrate-binding modules. *Curr. Opin. Struct. Biol.* **23**, 669–677
61. Armenta, S., Moreno-Mendieta, S., Sánchez-Cuapio, Z., Sánchez, S., and Rodríguez-Sanoja, R. (2017) Advances in molecular engineering of carbohydrate-binding modules. *Proteins Struct. Funct. Bioinforma.* **85**, 1602–1617
62. L. Robinson, S., Piel, J., and Sunagawa, S. (2021) A roadmap for metagenomic enzyme discovery. *Nat. Prod. Rep.* **38**, 1994–2023
63. Borkakoti, N., Ribeiro, A. J. M., and Thornton, J. M. (2025) A structural perspective on enzymes and their catalytic mechanisms. *Curr. Opin. Struct. Biol.* **92**, 103040
64. Buller, R., Lutz, S., Kazlauskas, R. J., Snajdrova, R., Moore, J. C., and Bornscheuer, U. T. (2023) From nature to industry: Harnessing enzymes for biocatalysis. *Science*. **382**, eadh8615
65. Wiltschi, B., Cernava, T., Dennig, A., Galindo Casas, M., Geier, M., Gruber, S., Haberbauer, M., Heidinger, P., Herrero Acero, E., Kratzer, R., Luley-Goedl, C., Müller, C. A., Pitzer, J., Ribitsch, D., Sauer, M., Schmölzer, K., Schnitzhofer, W., Sensen, C. W., Soh, J., Steiner, K., Winkler, C. K., Winkler, M., and Wriessnegger, T. (2020) Enzymes revolutionize the bioproduction of value-added compounds: From enzyme discovery to special applications. *Biotechnol. Adv.* **40**, 107520
66. Inoue, M., Yakushiji, T., Katsuki, M., Kudo, N., and Koga, T. (1988) Reduction of the adherence of *Streptococcus sobrinus* insoluble α-d-glucan by endo-(1→3)-α-d-glucanase. *Carbohydr. Res.* **182**, 277–286
67. Simonson, L. G., Gaugler, R. W., Lamberts, B. L., and Reiher, D. A. (1982) Purification and properties of endo-1,3-α-D-glucanase from *Pseudomonas*. *BBA - Gen. Subj.* **715**, 189–195
68. Hasegawa, S., Nordin, J. H., and Kirkwood, S. (1969) Enzymes that hydrolyze fungal cell wall polysaccharides. *J. Biol. Chem.* **244**, 5460–5470
69. Fuglsang, C. C., Berka, R. M., Wahleithner, J. A., Kauppinen, S., Shuster, J. R., Rasmussen, G., Halkier, T., Dalbøge, H., and Henrissat, B. (2000) Biochemical analysis of recombinant fungal mutanases: A new family of α1,3-glucanases with novel carbohydrate-binding domains. *J. Biol. Chem.* **275**, 2009–2018
70. Ait-lahsen, H., Soler, S., Rey, M., Monte, E., and Llobell, A. (2001) An antifungal exo-alpha-1,3-glucanase (AGN13.1) from the biocontrol fungus *Trichoderma harzianum*. **67**, 5833–5839
71. Sanz, L., Montero, M., Redondo, J., Llobell, A., and Monte, E. (2005) Expression of an α-1,3-glucanase during mycoparasitic interaction of *Trichoderma asperellum*. *FEBS J.* **272**, 493–499
72. Garcia, I., Jim, D., Mart, V., and Dur, A. (2005) The α-glucanase Agn1p is required for cell separation in *Schizosaccharomyces pombe*. **97**, 569–576
73. Sipiczki, M., Balazs, A., Monus, A., Papp, L., Horvath, A., Sveiczer, A., and Miklos, I. (2014) Phylogenetic and comparative functional analysis of the cell-separation α-glucanase Agn1p in *Schizosaccharomyces*. *Microbiol. U. K.* **160**, 1063–1074
74. Mazurkewich, S., Widén, T., Karlsson, H., Evenäs, L., Ramamohan, P., Wohlert, J., Brändén, G., and Larsbrink, J. (2025) Structural and biochemical basis for activity of *Aspergillus nidulans* α-1,3-glucanases from glycoside hydrolase family 71. *Commun. Biol.* **8**, 1298
75. He, X., Li, S., and Kaminskyj, S. (2017) An amylase-like protein, AmyD, is the major negative regulator for α-glucan synthesis in *Aspergillus nidulans* during the asexual life cycle. *Int. J. Mol. Sci.* **18**, 695
76. Wei, H., Scherer, M., Singh, A., Liese, R., and Fischer, R. (2001) *Aspergillus nidulans* α- 1,3 glucanase (mutanase), mutA, is expressed during sexual development and mobilizes mutan. *Fungal Genet. Biol.* **34**, 217–227
77. Grün, C. H., Dekker, N., Nieuwland, A. A., Klis, F. M., Kamerling, J. P., Vliegenthart, J. F. G., and Hochstenbach, F. (2006) Mechanism of action of the endo-(1 → 3)-α-glucanase MutAp from the mycoparasitic fungus *Trichoderma harzianum*. *FEBS Lett.* **580**, 3780–3786

78. Horaguchi, Y., Saitoh, H., Konno, H., Makabe, K., and Yano, S. (2025) Crystal structure of GH71 α-1,3-glucanase Agn1p from *Schizosaccharomyces pombe*: an enzyme regulating cell division in fission yeast. *Biochem. Biophys. Res. Commun.* **766**, 151907
79. Wiater, A., Szczodrak, J., Pleszczynska, M., and Próchniak, K. (2005) Production and use of mutanase from *Trichoderma harzianum* for effective degradation of streptococcal mutans. *Braz. J. Microbiol.* **36**, 137–146
80. Zonneveld, B. J. M. (1971) A new type of enzyme, an exo-splitting α-1,3-glucanase from non-induced cultures of *Aspergillus nidulans*. **258**, 541–547
81. Bauer, S., Vasu, P., Persson, S., Mort, A. J., and Somerville, C. R. (2006) Development and application of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 11417–11422
82. Shalom, G., Pratten, J., Wilson, M., and Nair, S. P. (2008) Cloning, heterologous gene expression and biochemical characterization of the α-1,3-glucanase from the filamentous fungus *Penicillium purpurogenum*. *Protein Expr. Purif.* **60**, 170–175
83. Hakamada, Y., Sumitomo, N., Ogawa, A., Kawano, T., Saeki, K., Ozaki, K., Ito, S., and Kobayashi, T. (2008) Nucleotide and deduced amino acid sequences of mutanase-like genes from Paenibacillus isolates: Proposal of a new family of glycoside hydrolases. *Biochimie*. **90**, 525–533
84. Pleszczyńska, M., Boguszewska, A., Tchórzewski, M., Wiater, A., and Szczodrak, J. (2012) Gene cloning, expression, and characterization of mutanase from *Paenibacillus curdlanolyticus* MP-1. *Protein Expr. Purif.* **86**, 68–74
85. Itoh, T., Panti, N., Hayashi, J., Toyotake, Y., and Matsui, D. (2020) Crystal structure of the catalytic unit of thermostable GH87 a -1 , 3- glucanase from *Streptomyces thermodiastaticus* strain HF3-3. *Biochem. Biophys. Res. Commun.* **533**, 1170–1176
86. Yano, S., Suyotha, W., Oguro, N., Matsui, T., Shiga, S., Itoh, T., Hibi, T., Tanaka, Y., Wakayama, M., and Makabe, K. (2019) Crystal structure of the catalytic unit of GH 87-type α-1,3-glucanase Agl-KA from *Bacillus circulans*. *Sci. Rep.* **9**, 1–10
87. Takahashi, M., Yano, S., Horaguchi, Y., Otsuka, Y., Suyotha, W., Makabe, K., Konno, H., and Kokeguchi, S. (2023) α-1,3-Glucanase from the gram-negative bacterium *Flavobacterium* sp. EK-14 hydrolyzes fungal cell wall α-1,3-glucan. *Sci. Rep.* **13**, 21420
88. Okazaki, K., Amano, T., Morimoto, T., Iemoto, T., Kawabata, T., Hayakawa, S., and Akimitsu, K. (2001) Cloning and nucleotide sequence of the mycodextranase gene from *Streptomyces* sp. J-13-3. *Biosci. Biotechnol. Biochem.* **65**, 1684–1687
89. Itoh, T., Intuy, R., Suyotha, W., Hayashi, J., Yano, S., Makabe, K., Wakayama, M., and Hibi, T. (2020) Structural insights into substrate recognition and catalysis by glycoside hydrolase family 87 α-1,3-glucanase from *Paenibacillus glycanilyticus* FH11. *FEBS J.* **287**, 2524–2543
90. Suyotha, W., Yano, S., Itoh, T., Fujimoto, H., Hibi, T., Tachiki, T., and Wakayama, M. (2014) Characterization of α-1,3-glucanase isozyme from *Paenibacillus glycanilyticus* FH11 in a new subgroup of family 87 α-1,3-glucanase. *J. Biosci. Bioeng.* **118**, 378–385
91. Suyotha, W., Yano, S., Takagi, K., Rattanakit-Chandet, N., Tachiki, T., and Wakayama, M. (2013) Domain structure and function of α-1,3-glucanase from *Bacillus circulans* KA-304 , an enzyme essential for degrading basidiomycete cell walls. **77**, 639–647
92. Abbott, D. W., Ficko-Blean, E., van Bueren, A. L., Rogowski, A., Cartmell, A., Coutinho, P. M., Henrissat, B., Gilbert, H. J., and Boraston, A. B. (2009) Analysis of the structural and functional diversity of plant cell wall specific family 6 carbohydrate binding modules. *Biochemistry*. **48**, 10395–10404
93. Panti, N., Cherdvorapong, V., Itoh, T., Hibi, T., Suyotha, W., Yano, S., and Wakayama, M. (2021) Functional analysis of α-1,3-glucanase domain structure from *Streptomyces thermodiastaticus* HF3-3. *J. Gen. Appl. Microbiol.* **67**, 85–91
94. Correia, M. A. S., Abbott, D. W., Gloster, T. M., Fernandes, V. O., Prates, J. A. M., Montanier, C., Dumon, C., Williamson, M. P., Tunnicliffe, R. B., Liu, Z., Flint, J. E., Davies, G. J., Henrissat, B., Coutinho, P. M., Fontes, C. M. G. A., and Gilbert, H. J. (2010) Signature active site architectures illuminate the molecular basis for ligand specificity in family 35 carbohydrate binding module,. *Biochemistry*. **49**, 6193–6205
95. Fujimoto, Z., Suzuki, N., Kishine, N., Ichinose, H., Momma, M., Kimura, A., and Funane, K. (2017) Carbohydrate-binding architecture of the multi-modular α-1,6-glucosyltransferase from Paenibacillus sp. 598K, which produces α-1,6-glucosyl-α-glucosaccharides from starch. *Biochem. J.* **474**, 2763–2778
96. Suzuki, N., Fujimoto, Z., Kim, Y.-M., Momma, M., Kishine, N., Suzuki, R., Suzuki, S., Kitamura, S., Kobayashi, M., Kimura, A., and Funane, K. (2014) Structural elucidation of the cyclization mechanism of α-1,6-glucan by *Bacillus circulans* T-3040 cycloisomaltooligosaccharide glucanotransferase. *J. Biol. Chem.* **289**, 12040–12051
97. Bolam, D. N., Xie, H., Pell, G., Hogg, D., Galbraith, G., Henrissat, B., and Gilbert, H. J. (2004) X4 modules represent a new family of carbohydrate-binding modules that display novel properties. *J. Biol. Chem.* **279**, 22953–22963

98. Abbott, D. W., and Van Bueren, A. L. (2014) Using structure to inform carbohydrate binding module function. *Curr. Opin. Struct. Biol.* **28**, 32–40
99. Michel, G., Barbeyron, T., Kloareg, B., and Czjzek, M. (2009) The family 6 carbohydrate-binding modules have coevolved with their appended catalytic modules toward similar substrate specificity. *Glycobiology*. **19**, 615–623
100. Abbott, D. W., Eirín-López, J. M., and Boraston, A. B. (2008) Insight into ligand diversity and novel biological roles for family 32 carbohydrate-binding modules. *Mol. Biol. Evol.* **25**, 155–167
101. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410
102. Blaber, M. (2022) Variable and conserved regions of secondary structure in the β-trefoil fold: structure versus function. *Front. Mol. Biosci.* 10.3389/fmolb.2022.889943
103. Notenboom, V., Boraston, A. B., Williams, S. J., Kilburn, D. G., and Rose, D. R. (2002) High-resolution crystal structures of the lectin-like xylan binding domain from *Streptomyces lividans* xylanase 10A with bound substrates reveal a novel mode of xylan binding. *Biochemistry*. **41**, 4246–4254
104. Miyanaga, A., Koseki, T., Matsuzawa, H., Wakagi, T., Shoun, H., and Fushinobu, S. (2004) Crystal structure of a family 54 α-l-arabinofuranosidase reveals a novel carbohydrate-binding module that can bind arabinose. *J. Biol. Chem.* **279**, 44907–44914
105. Hao, M.-S., Mazurkewich, S., Li, H., Kvammen, A., Saha, S., Koskela, S., Inman, A. R., Nakajima, M., Tanaka, N., Nakai, H., Brändén, G., Bulone, V., Larsbrink, J., and McKee, L. S. (2024) Structural and biochemical analysis of family 92 carbohydrate-binding modules uncovers multivalent binding to β-glucans. *Nat. Commun.* **15**, 3429
106. Suyotha, W., Yano, S., and Wakayama, M. (2016) α-1,3-Glucanase: present situation and prospect of research. *World J. Microbiol. Biotechnol.* **32**, 1–11
107. Kaplan, J. B. (2010) Biofilm dispersal. *J. Dent. Res.* **89**, 205–218
108. Klein, M. I., Xiao, J., Lu, B., Delahunty, C. M., Yates, J. R., and Koo, H. (2012) *Streptococcus mutans* protein synthesis during mixed-species biofilm development by high-throughput quantitative proteomics. *PLoS ONE*. **7**, e45795
109. Sumitomo, N., Saeki, K., Ozaki, K., Ito, S., and Kobayashi, T. (2007) Mutanase from a Paenibacillus isolate: Nucleotide sequence of the gene and properties of recombinant enzymes. *Biochim. Biophys. Acta - Gen. Subj.* **1770**, 716–724
110. Tsumori, H., Kawauti, T., Shimamura, A., Hanada, N., Sakurai, Y., and Yamakami, K. (2010) Cloning and expression of the mutanase gene of Paenibacillus humicus from fermented food. *J. Health Sci.* **56**, 456–461
111. McKee, L. S., La Rosa, S. L., Westereng, B., Eijsink, V. G., Pope, P. B., and Larsbrink, J. (2021) Polysaccharide degradation by the Bacteroidetes: mechanisms and nomenclature. *Environ. Microbiol. Rep.* 10.1111/1758-2229.12980
112. Izadi, H., Asadi, H., and Bemani, M. (2025) Chitin: a comparison between its main sources. *Front. Mater.* 10.3389/fmats.2025.1537067
113. Hu, X.-Q., Xia, B.-B., Ru, W.-J., Zhang, Y.-X., Yang, J.-W., and Zhang, H.-B. (2023) Research progress on structure and catalytic mechanism of dextranase. *eFood*. **4**, e60
114. Widén, T., Rangel, A. T., Lombard, V., Drula, E., Mazurkewich, S., Terrapon, N., Kerkhoven, E. J., and Larsbrink, J. (2024) *Streptomyces castrisilvae* sp. nov. and *Streptomyces glycanivorans* sp. nov., novel soil streptomycetes metabolizing mutan and alternan. *Int. J. Syst. Evol. Microbiol.* 10.1099/ijsem.0.006514
115. Nikolaidis, M., Hesketh, A., Frangou, N., Mossialos, D., Van de Peer, Y., Oliver, S. G., and Amoutzias, G. D. (2023) A panoramic view of the genomic landscape of the genus *Streptomyces*. *Microb. Genomics*. **9**, 1028
116. Chater, K. F., Biró, S., Lee, K. J., Palmer, T., and Schrempf, H. (2010) The complex extracellular biology of *Streptomyces*. *FEMS Microbiol. Rev.* **34**, 171–198
117. Decker, S. R., Adney, W. S., Vinzant, T. B., and Himmel, M. E. (2003) Alkaline tolerant dextranase from *Streptomyces anulatus*. [online] https://patents.google.com/patent/US6509184B1/en (Accessed December 15, 2025)
118. Cologna, N. de M. di, Gómez-Mendoza, D. P., Zanoelo, F. F., Giannesi, G. C., Guimarães, N. C. de A., Moreira, L. R. de S., Filho, E. X. F., and Ricart, C. A. O. (2018) Exploring *Trichoderma* and *Aspergillus* secretomes: Proteomics approaches for the identification of enzymes of biotechnological interest. *Enzyme Microb. Technol.* **109**, 1–10
119. Bengtsson, O., Arntzen, M., Mathiesen, G., Skaugen, M., and Eijsink, V. G. H. (2016) A novel proteomics sample preparation method for secretome analysis of *Hypocrea jecorina* growing on insoluble substrates. *J. Proteomics*. **131**, 104–112
120. Tuveng, T. R., Arntzen, M. Ø., Bengtsson, O., Gardner, J. G., Vaaje-Kolstad, G., and Eijsink, V. G. H. (2016) Proteomic investigation of the secretome of *Cellvibrio japonicus* during growth on chitin. *PROTEOMICS*. **16**, 1904–1914
121. O. Sheridan, P., Martin, J. C., Lawley, T. D., Browne, H. P., Harris, H. M. B., Bernalier-Donadille, A., Duncan, S. H., O'Toole, P. W., P. Scott, K., and J. Flint, H. (2016) Polysaccharide utilization loci and

nutritional specialization in a dominant group of butyrate-producing human colonic Firmicutes. *Microb. Genomics*. **2**, e000043

122. Titgemeyer, F., Reizer, J., Reizer, A., and Saier, M. H. (1994) Evolutionary relationships between sugar kinases and transcriptional repressors in bacteria. *Microbiology*. **140**, 2349–2354
123. Berntsson, R. P.-A., Smits, S. H. J., Schmitt, L., Slotboom, D.-J., and Poolman, B. (2010) A structural classification of substrate-binding proteins. *FEBS Lett.* **584**, 2606–2617
124. Hall, K. R., Mollatt, M., Forsberg, Z., Golten, O., Schwaiger, L., Ludwig, R., Ayuso-Fernández, I., Eijsink, V. G. H., and Sørlie, M. (2024) Impact of the copper second coordination sphere on catalytic performance and substrate specificity of a bacterial lytic polysaccharide monooxygenase. *ACS Omega*. **9**, 23040–23052
125. Forsberg, Z., Bissaro, B., Gullesen, J., Dalhus, B., Vaaje-Kolstad, G., and Eijsink, V. G. H. (2018) Structural determinants of bacterial lytic polysaccharide monooxygenase functionality. *J. Biol. Chem.* **293**, 1397–1412
126. Kobayashi, K., Hasegawa, T., Kusumi, R., Kimura, S., Yoshida, M., Sugiyama, J., and Wada, M. (2017) Characterization of crystalline linear (1 → 3)-α-d-glucan synthesized *in vitro*. *Carbohydr. Polym.* **177**, 341–346
127. Nakamura, S., Kurata, R., Tonozuka, T., Funane, K., Park, E. Y., and Miyazaki, T. (2023) Bacteroidota polysaccharide utilization system for branched dextran exopolysaccharides from lactic acid bacteria. *J. Biol. Chem.* **299**, 104885
128. Nakamura, S., Funane, K., and Miyazaki, T. (2025) Identification and characterization of an endodextranase from *Flavobacterium johnsoniae*, the first endo-acting enzyme classified within glycoside hydrolase family 27. *Biosci. Biotechnol. Biochem.* 10.1093/bbb/zbaf130
129. Huang, Y., Liu, L., Wang, R., Jiang, T., Yu, Q., Wang, E., and Yuan, H. (2024) Functional identification of carbohydrate-binding module 13 and its application to quantification of hemicellulose in gramineous plants. *Int. J. Biol. Macromol.* **282**, 136752
130. Fujikawa, T., Sakaguchi, A., Nishizawa, Y., Kouzai, Y., Minami, E., Yano, S., Koga, H., Meshi, T., and Nishimura, M. (2012) Surface α-1,3-glucan facilitates fungal stealth infection by interfering with innate immunity in plants. *PLOS Pathog.* **8**, e1002882
131. Calo, L., García, I., Gotor, C., and Romero, L. C. (2006) Leaf hairs influence phytopathogenic fungus infection and confer an increased resistance when expressing a *Trichoderma* α-1,3-glucanase. *J. Exp. Bot.* **57**, 3911–3920
132. Hoffstedt, T., Skov Hansen, L. B., Twetman, S., and Sonesson, M. (2022) Effect of an enzyme-containing mouthwash on the dental biofilm and salivary microbiome in patients with fixed orthodontic appliances: a randomized placebo-controlled pilot trial. *Eur. J. Orthod.* 10.1093/ejo/cjac062
133. Singh, R., Ren, Z., Shi, Y., Lin, S., Kwon, K.-C., Balamurugan, S., Rai, V., Mante, F., Koo, H., and Daniell, H. (2021) Affordable oral health care: dental biofilm disruption using chloroplast made enzymes with chewing gum delivery. *Plant Biotechnol. J.* **19**, 2113–2125
134. Del Rey, Y. C., Parize, H., Assar, S., Göstemeyer, G., and Schlafer, S. (2024) Effect of mutanase and dextranase on biofilms of cariogenic bacteria: A systematic review of *in vitro* studies. *Biofilm*. **7**, 100202
135. Otsuka, Y., Sasaki, K., Suyotha, W., Furusawa, H., Miyazawa, K., Konno, H., and Yano, S. (2022) Construction of a fusion protein consisting of α-1,3-glucan-binding domains and tetrameric red fluorescent protein, which is involved in the aggregation of α-1,3-glucan and inhibition of fungal biofilm formation. *J. Biosci. Bioeng.* **133**, 524–532