Identification of the Genes Encoding Enzymes Involved in the Synthesis of the Biopolymer Paramylon from *Euglena gracilis*

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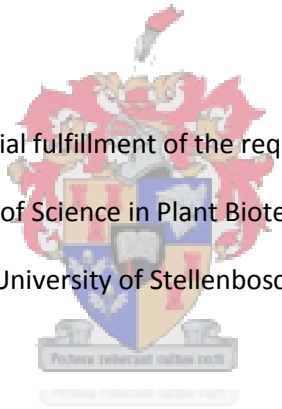

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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December 2010

Abstract

Recent advances in medical pharmacology have identified the immune-potentiating effects of β-1,3-glucans on mammalian immune systems. Extensive research has identified and described the mechanisms of action and receptor binding specificity of different β-1,3-glucans as well as their structural and functional relationships. Molecular mass, solubility, structural order, degree of branching as well as chemical modification all determine the effectiveness of the β-1,3-glucan immune-modulating activities, which typically include; macrophage activation, antibody adjuvant activities, reduction of LDL-cholesterol, leukocyte mitogenic activities, cytokine and chemokine production as well as antiviral and antitumor activities. Currently β-1,3-glucans have been sold commercially under the name β-glucan, mostly in the form of Betafectin, a genetically modified yeast derived β-1,3-glucan.

Recent studies of different β-1,3-glucans have identified the pharmacological activities of paramylon, a *Euglena* derived β-1,3-glucan. Although paramylon has relatively low immune-stimulating activities, chemical modification of the paramylon granule increased immune-potentiation with specific antimicrobial and anti-HIV activities. Due to these specific immune-potentiating activities, paramylon is novel in terms of both structure as well as functional activity.

In terms of biotechnological application, paramylon is greatly favoured as it is synthesized as an insoluble membrane bound granule in the cytosol of *Euglena* where most plant and fungal β-1,3-glucan synthases are cell membrane bound highly regulated multifunctional complexes, synthesizing β-1,3-glucan as cell wall components. Due to the novel granular nature of paramylon, expression in other systems with genetic modification could potentially further increase immuno-potentiating activities.

In this study, different approaches were attempted in order to identify the genes involved in paramylon synthesis including; constructing and screening a *Euglena gracilis* cDNA library, sequence analysis of the purified proteins as well as transcription analysis of the sequenced transcriptome and genome of *E. gracilis*. Putative candidates that encode subunits of the paramylon synthase complex have been identified.

Opsomming

Onlangse vordering in mediese farmakologie het die immuun-stimulerende effek van β-1,3-glukane op die soogdier immuunsisteem geïdentifiseer. Intensiewe navorsing het die meganisme van die werking en reseptor bindingspesifisiteit van verskillende β-1,3-glukane, asook hulle struktuur en funksionele verhoudings, geïdentifiseer. Die molekulêre massa, oplosbaarheid, strukturele orientasie, mate van vertakking asook chemiese modifikasies bepaal almal die effektiwiteit van die β-1,3-glukaan immuun-modulerende aktiwiteite. Tipiese immuno-moduleringsaktiwiteite sluit makrofaag aktivering, teenliggaampie adjuvant aktiwiteite, verlaging van LDL-cholesterol, leukosiet mitogeniese aktiwiteite, sitokien en chemokien produksie asook anti-virale en antitumor aktiwiteite in. Huidiglik word β-1,3-glukane onder die naam β-glukaan verkoop meestal in die vorm van Betafectin, 'n geneties gemodifiseerde gis wat van β-1,3-glukaan afkomstig is.

Onlangse studies van verskillende β-1,3-glukane het die farmakologiese aktiwiteit van paramylon, 'n Euglena afkomstige β-1,3-glukaan geïdentifiseer. Alhoewel paramylon relatiewe lae immuun-stimulerende aktiweite toon, verhoog chemiese modifikasies van die paramylon granules immuun-stimulering, spesifiek die anti-mikrobiese en anti-MIV aktiwiteite. Weens hierdie spesifieke immuun-stimulerende aktiweite, word paramylon as nuut beskou veral in terme van beide struktuur asook funksionele aktiwiteit.

In terme van biotegnologiese toepassing, verkry paramylon voorkeur aangesien dit as 'n onoplosbare membraangebonde granule in die sitosol van Euglena gesintetiseer word terwyl meeste plant en fungus β-1,3-glukaan sintases hoogs gereguleerde multifunksionele selmembraan gebonde komplekse is wat β-1,3-glukaan asook ander selwand komponente sintetiseer. Weens die unieke granulêre natuur van paramylon, sal uitdrukking in ander sisteme 'n moontlike industrie skep waar deur die transgeniese uitdrukking van granulêre paramylon verdere verbetering van die immuun-stimulerings aktiwiteite kan lei.

In hierdie studie is verskillende benaderings aangewend om die gene wat by paramylon sintese betrokke is te identifiseer, dit sluit in die konstruksie en sifting van 'n *E. gracilis* cDNS biblioteek, aminosuur volgorde analise van gesuiwerde proteiene asook die transkripsionele analise van die volgorde van die transkriptoom en genoom van *E. gracilis*. Moontelike kandidate wat vir die subeenhede van die paramylon syntase kompleks kodeer is geïdentifiseer.

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Abbreviations

^{0}C	degrees centigrade
^{14}C	Carbon-14 radio-isotope
bp	base pairs
BSA	Bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-cholamidopropyl)-dimethylammino]-1-propane sulfonate
CNBr	cyanogen bromide
CTAB	cetyltrimethylammonium bromide
DMSO	dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNAse I	Deoxyribonuclease I (EC 3.1.21.1)
DTT	dithiotreitol
E- value	Expect value
EDTA	ethylenediaminetetraacetic aid
EST	expressed sequence tag
FPLC	flow pressure liquid chromatography
g	gram
g	gravitational force
GT	glycosyl transferase
GTP	guanidine triphosphate
h	hour
IPTG	isopropyl- β-D-thiogalactopyranoside
kDa	kilo Dalton
L	litre
LB	Luria broth

LCMSMS	liquid chromatography mass spectrophotometry
mg	milligram
min	minute
ml	milliliter
µl	microlitre
µM	micromolar
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
m/v	mass per volume
nm	nanometers
OD_{600}	optical density at 600 nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
s	second
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
TEMED	N,N,N'N'-tetramethylethylenediamine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
U	enzyme units
UDP	uridine diphosphate
v/v	volume per volume
w/v	weight per volume

Review of Literature

1.1 *Euglena gracilis*

Euglenoids (Euglenophyceae, Euglenophyta), consist of around 40 genera and 1000 species (van den Hoek *et al*., 1995). *Euglena*, the most common fresh water genus, are autotrophs, although some exist as obligate hetrotrophs grow normally as spindle-shape cylindrical cells and can vary in length between 10 µm and 500 µm. Euglenids have been difficult to place phylogenetically due to their unusual morphology. The photoautotrophic euglenoids and the secondary hetrotrophs are characterized by their specific pellicle, paramylon, a β-1,3-glucan (Barras and Stone, 1968), as well as the euglenoid specific closed mitosis. In phototrophs, *Euglenae* are characterized by chloroplasts enveloped by three membranes which contain chlorophylls *a* and *b,* β-carotene, antheroxanthin, neoxanthin and catanoid and quinones (Leedale, 1967), a phototactic photoreceptor (Dawson and Walne 1991b) and a separate extraplastidial eyespot (Kreimer, 1994; Leedale, 1967; Walne and Arnott, 1967; Walne and Kivic, 1990).

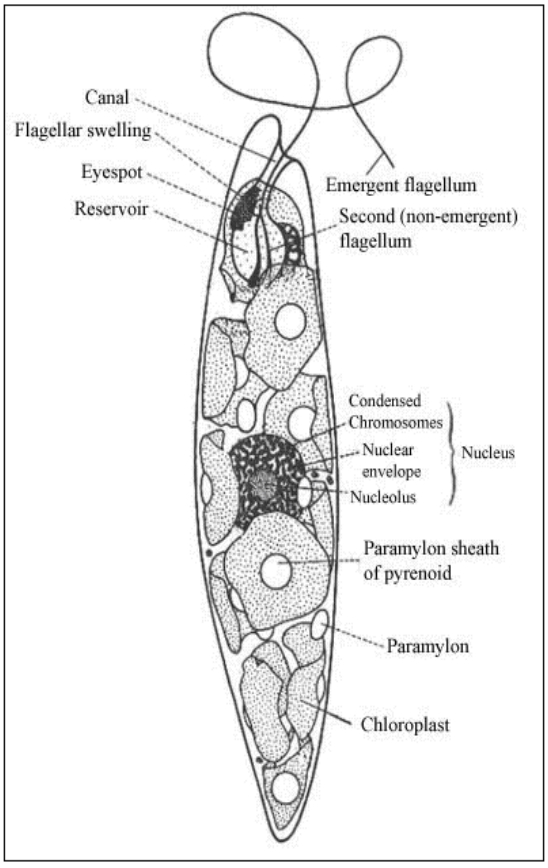

Figure 1: *Euglena graclis.* (www.botany.ubc.ca/Biol320/ultra/whol03.htm)

Besides paramylon synthesis, *E. gracilis* (Figure 1) is one of the few microorganisms which synthesize high amounts of antioxidant vitamins such as β-carotene, L-ascorbic acid and α-tocopherol simultaneously (Takeyama *et al*., 1997) and produce relatively large amounts of wax esters (Inui *et al*., 1983) and highly unsaturated fatty acids (Hayashi *et al*., 1993) which is valued in the food and feed industries (Kitaoka and Hosotani, 1977). *Euglena* naturally rely on decaying organic matter as food source and grow on media consisting of acetate, glucose, glutamate, succinate, pyruvate and even high concentrations of ethanol in light and dark conditions as well as ammonia (Kusmic *et al*., 1999) and are able to grow under harsh acid conditions as low as pH 1.8 (Yamane *et al*., 2001).

1.2 Taxonomy

Euglenophyceae (Euglenazoa) are part of a diverse group of flagellate protists consisting of *euglenoids*, *kinetoplastids*, *diplonemids* and *postgaardi* all identified and characterized by the unique root pattern and paraxonemal cylinders in the flagella (Fenchel *et al*. 1995; Patterson, 1999; Simpson, 1997; Simpson *et al*. 1997). Cavalier-Smith (2003a, 2003b) placed the phylum Euglenazoa in the kingdom of Protozoa, subkingdom Bicilata and intrakingdom Excavata. Photosynthetic euglenoids and secondary hetrotrophs form the Euglenophyceae clade with two orders: Euglenales and Eutreptiales. Marine Eutreptiales (Eutreptia and Eutreptiella) consist of biflagellate and quadriflagellate taxa. Fresh water Euglenales form nine clades and two individual branches. *Euglena* are polyphyletic and split into four independent clades and two individual branches.

Most photoautotrophic eukaryotes inherited photosynthetic abilities from a cyanobacterial endosymbiont (McFadden, 2001), from which the genome of the endosymbiont was reduced to a plasmid within the chloroplasts of red and green algae, glaucocystophytes and higher plants (Adl *et al*., 2005). While several other independent eukaryotic lineages adopted photosynthetic capabilities from another secondary endosymbiotic event where a eukaryotic algae was engulfed by the host eukaryote (Gibbs, 1978; Stoebe and Maier, 2002). Where both endosymbiotic events resulted in endosymbiotic gene transfer between host chromosomes and organelles (Archibald *et al*., 2003; Martin *et al*., 1993; Timmis *et al*., 2004). Endosymbiotic gene transfer can vary in frequency from primary endosymbiosis of 11% in *Cyanophora* (Reyes-Prieto *et al*., 2006) and 18% in *Arabadopsis thaliana* (Martin *et al*., 2002).

E. gracilis shares a common ancestor with Kinetoplastida (*Trypanosoma*) (Adl *et al*., 2005), which have never undergone secondary endosymbiosis (Rogers *et al*., 2006). The *E. gracilis* plastid was acquired from a secondary endosymbiotic event although without traces of the secondary endosymbiont nucleus (McFadden, 2001). Judging from similarities in sequence analysis, *E. gracilis* appears to be a hybrid with a genome composed of Euglena-specific genes, Kinetoplastida-specific genes as well as common eukaryote

genes and genes acquired from the secondary endosymbiotic event most likely of photoautotrophic eukaryote origin having othologues with only photoautotrophic eukaryotes (Henze *et al*., 1995). About 14% of the genes have orthologues limited to photoautotrophic eukaryotes of which 25 % are related to photosynthesis while only 4% are derived from Kinetoplastida, most of which are unknown open reading frames (Ahmadinejad *et al*., 2007).

Due to the horizontal gene transfer of the secondary endosymbiotic events it has been difficult to describe *Euglena* in a phylogenetic tree as the hybrid genome is derived from two ancestors (Dagan and Martin, 2006). Currently *Euglena* have been distributed in a monophyletic clade with Kinetoplastida although this only accounts for the Kinetoplastid ancestor and not the secondary endosymbiotic event (Figure 2) (Ahmadinejad *et al*., 2007).

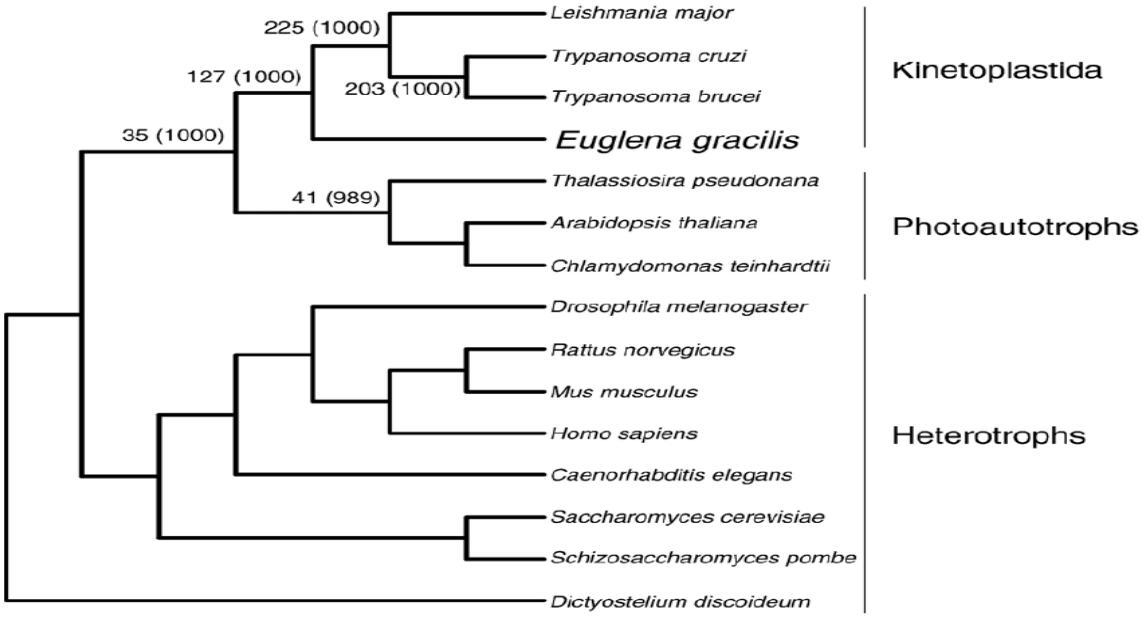

Figure 2: The phylogeny of *E. gracilis* where 259 globally distributed genes distributed in a phylogenetic tree grouped E. gracilis in a monophyletic clade with Kinetoplastids where the secondary endosymbiotic event is not represented (Ahmadinejad et al., 2007).

1.3 Paramylon and paramylon synthesis

β-1,3-glucans are common in the natural world where they serve as structural frameworks, in the form of callose in plants (Samuels *et al*., 1995), primary cell walls in fungi (Cabib *et al*., 1987) and as exopolysaccharides in bacteria (Marchessault and Desandes., 1979). β-1,3-glucans exist as storage carbohydrates in the Phaecophyceae, Chrysophyceae and in Euglenaphyceae (De Madariaga., 1992; Kiss

and Triemer., 1988; Kreeger and Van der Veer., 1970) as well as Heterokontophyta and Haptophyta (van den Hoek., 1995).

In most organisms that store β-1,3-glucans as carbohydrate reserve, the glucan polymers are non-granular as opposed to cytosolic granular *Euglenoid* paramylon (Barras and Stone., 1968). The highly crystalline substructure of granular paramylon is unique to *Euglena* (Marchessault and Deslandes, 1979), where the paramylon β-1,3-glucan chains are arranged in triple helical microfibrils arranged from a central region which connects the segments of the granule in a concentric pattern (Booy *et al*., 1981; Deslandes and Marchessault, 1980; Marchessault and Deslandes., 1979).

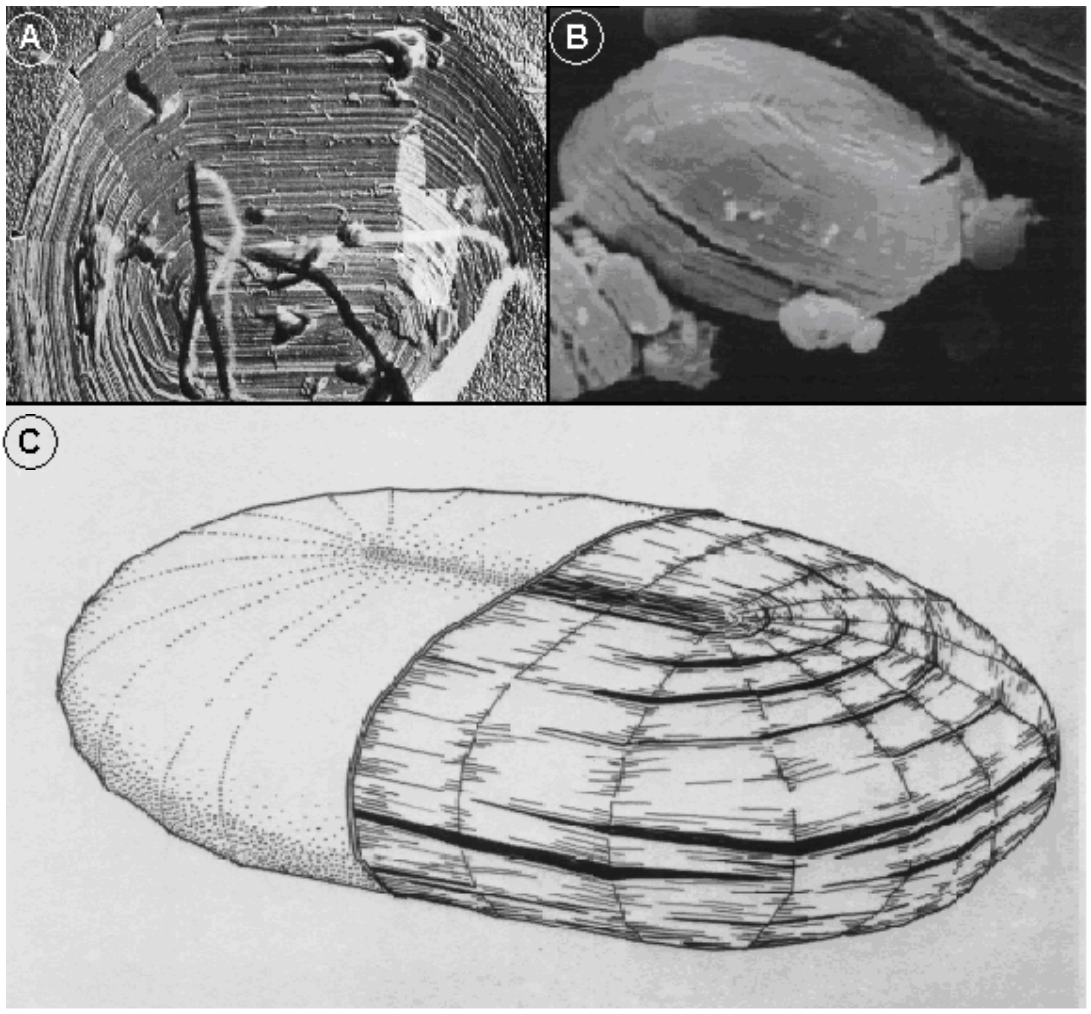

Figure 3: The structure of the paramylon granule. SEM of the paramylon granule from *E. gracilis* (A) (Kiss et al., 1987), SEM freeze fractured paramylon granule (B) (Kiss et al., 1987) and a diagrammatic representation of a paramylon granule surrounded by the membrane bilayer (C) (Kiss et al., 1987).

The triangular segments are arranged in three dimensional wedges, while the rectangular segments are block-shaped (Figure 3a). The microfibrils are arranged in complex segments with the layered mature microfibrils are arranged in the centre surrounded concentrically by maturing microfibrils (Figure 3a) (Barras and Stone., 1968; Kiss *et al*., 1987; Marchessasault and Deslandes, 1979) similar to the microfibril structure of cellulose (Gardner and Blackwell., 1971; Kato, 1981). The microfibrils are enveloped by a bilayer lipid membrane (Kiss *et al*., 1988) which has large intramembraneous particles, which increase in density with granule synthesis (Figures 3b and 3c) (Kiss *et al*., 1988). Paramylon was shown to be first synthesized as elementary microfibrils with low chrystalinity (Kiss *et al*., 1987, 1988a). As the granule matures the microfibrils assemble to form a highly ordered crystalline structure (Booy *et al*., 1981; Kiss *et al*., 1988a; Marchessault and Deslanders., 1979).

Paramylon granules are synthesized by membrane bound β-1,3-glycosyl transferases using UDP-glucose as the substrate (Marchessault and Deslandes., 1979) and are located cytosolically, surrounding pyrenoids in autotrophic cells and distributed freely at a 6-fold greater amount in heterotrophic cell cytosols (Briand and Calvayrac., 1980; Kiss *et al*., 1986, 1988; Marechal and Goldberg., 1964; Tomos and Northcote, 1978). A protein primer is thought to be responsible for initial formation of the paramylon granule where the protein-glucan intermediate consists of glucan bound to an aglycone, partly by a phosphate ester bond, although the responsible protein has not yet been identified (Tomos and Northcote., 1978). A similar self glycosylating protein, a UDP-glucose glycosyl transferase, has been observed in plants (Quentmeier *et al*., 1987).

The membrane surrounding the paramylon granule is distinctive as most other storage granules are not membrane bound (Kiss *et al*., 1987). Freeze-etch fractionation of the membrane revealed two types of intramembraneous particles associated with the membrane, a large type of intramembraneous particles and smaller one. The membrane and intramembraneous particles are always associated with the granule and it is expected that new membranes are synthesized with the new granules as well as associated structural proteins (Kiss *et al*., 1987). The frequency of the intramembraneous particles observed were related directly to paramylon synthesis and thought to be functional in paramylon synthesis (Kiss *et al*., 1987). Other intramembraneous particles have been identified as transport proteins (Blaurock and Stoekenius, 1971; Henderson and Unwin, 1975) or related to cellulose synthesis (Mueller and Brown, 1980; Quader, 1986; Staehelin and Giddings, 1982).

The large and small intramembraneous particles both seem to be involved in paramylon synthesis where the large intramembraneous particles are more frequent during early stages of the granule synthesis and the smaller intramembraneous particle predominant in later stages of granule development. The larger intramembraneous particle may be the paramylon synthase complexes themselves while the smaller may be a transport or structural protein or involved in degradation (Kiss *et al*., 1987). The high degree of

similarity between paramylon granules and the crystalline nature of cellulose suggest a similar mechanism of paramylon synthesis to cellulose synthesis where cellulose is synthesized by a membrane bound regulated multi-subunit enzyme complex (Delmer, 1999; Marchessault and Deslandes, 1979; Preston *et al*., 1964).

1.4 Glycosyl transferases

Glycosyl transferases are enzymes that catalyze the covalent transfer of sugar residues between donor substrates and their acceptor molecules where the donor molecules are sugar phosphates, di-, oligo or polysaccharides and nucleotide diphosphate sugars and the acceptor molecule is usually a carbohydrate polysaccharide, lipid carrier or protein (Hundle *et al*., 1992). Glycosyl transferases are sub-classed into two groups: processive glycosyl transferases such as cellulose, callose and chitin synthases that can transfer multiple sugar residues between donors and the acceptor polysaccharide while non-processive glycosyl transferases which transfer single sugar residues to their acceptor (Coutinho *et al*. 2003).

The vast majority of sequenced glycosyl transferases exist in the form of uncharacterized ORF's. Almost half of the sequenced glycosyl transferases are from sequenced genomes. Poly-specific families of glycosyl transferases have been included however glycosyl hydrolases and transglycosidases are not included in this system of classification as they are mechanistically and structurally unrelated (Henrissat, 1991; Henrissat and Bairoch, 1993; Henrissat and Bairoch, 1996). The greatest difficulties in glycosyl transferase characterization are due to multiple membrane-spanning domains and membrane associated activities (Laine, 1994). Substitutions of single amino acids may have significant impacts on glycosyl transferase activities such as substrate and product specificities where poly-specific families become difficult to predict with limited sequence information (Seto *et al*., 1999). Structurally, glycosyl transferases can adopt one of two fold conformations but due to the multiple substrate donors and wide range of acceptor molecules, glycosyl transferases are complex and diverse in primary structure making functional prediction very difficult with limited sequence information (Coutinho *et al*. 2003).

Common domain organizations and interrelationships have been identified in several glycosyl transferases based on sequence comparison (Paulson *et al*., 1989). Regions of distant sequence similarity have also been studied and identified (Atkinson and Long, 1992; Bulawa, 1992; De Angelis *et al*., 1994; Debellé *et al*., 1992; Dougherty and van de Rijn, 1994; Glucksmann *et al*., 1993; Saxena *et al*., 1994) suggesting that significant conserved domains are essential for the different glycosyl transferases. Very poor sequence similarities between glycosyl transferases have made global alignment impossible due to high divergence levels. However analysis aimed at secondary globular structures and transmembrane domains of the enzymes identified two conserved domains among glycosyl transferases where domain A, situated at the N terminal

half, is highly conserved and domain B, situated at the C terminal half, is conserved to a lesser extent. Glycosyl transferases consisting of both domains A and B appear to be processive glycosyl trasferases (β-glucan synthases) opposed to non-processive glycosyl transferases having only domain A (Saxena *et al*., 1995).

When glycosyl transferase amino acid sequences were searched for conserved amino acids having a side chain able to capably catalyze the acid-base transferase reaction, only two polar D residues were conserved among all the sequences (Zvelebil *et al*., 1988). Domain A has been further analyzed (Gaboriaud, 1987; Lemesle-Varloot *et al*., 1990) revealing vertically shaped clusters resembling β-strands alternating with α-helices where the D residues are invariably positioned in C terminal loops as expected with catalytic residues (Jacobson *et al*., 1994; Zvelebil *et al*., 1988). The hydrophobic clusters in domain B are more variable relative to domain A with only a single conserved D residue and the conserved QXXRW for cellulose synthases (Delmer, 1999; Saxena and Brown, 1999) or QXRRW for chitin synthases (Ruiz-Herrera *et al*., 2002) sequence motifs presumed to be involved in the catalytic reaction (Hieta *et al*., 1984). The bacterial β-1,3-glucan synthase genes share similar but not necessarily conserved domains where domain A shares the two D residues, one in the UDP-glucose binding domain as well as a KAG domain (YXXRXXXXHAKAGNLN) while domain B contains a TED domain with the third D residue and highly conserved QXXRW or QRXRW motifs (Stasionopoulus *et al*., 1999).

Distant similarities between families point towards evolutionary divergence although all glycosyl transferase families are separated into two folding patterns, GT-A and GT-B (Bourne and Henrissat, 2001; Coutinho *et al*. 2003; Hu and Walker, 2002; Tarbouriech *et al*., 2001; Ünligil *et al*., 2000) consisting of domain A, situated at the N terminal half and is highly conserved, and domain B, situated at the C terminal half, is conserved to a lesser extent (Saxena *et al*., 1995). GT-A consists of two β-α-β domains which form continuous central sheets of at least eight β-strands. GT-B consists of two β-α-β domains associated weakly where ligand binding is associated with conformational changes with no significant sequence similarities between families (Wrabl and Grishin, 2001). Nucleotide binding occurs on the N-terminal of the GT-A enzymes and at the C-terminal of the GT-B enzymes while acceptor binding occurs on the opposite domain. The folds themselves do not determine the stereochemical reaction.

Glycosyl transferases are further separated into families GT1-65 where enzymes within the family although structurally related are functionally distinct. β-1,3-glucan synthases fall specifically under two gene families: GT2, a GT-A folding clan I inverting glycosyl transferase which has a wide range across all taxonomic kingdoms and consist of cellulose synthase, chitin synthase, β-*N*-acetylglucosaminyltransferase, β-*N*-acetylgalactosaminyltransferase, β-mannosyl transferase, dolichyl phosphate, hyaluronan synthase and β-1,3-glucan synthase. While GT48, an inverting glycosyl transferase not yet completely characterized,

belongs to plant and fungal kingdoms where only the β-1,3-glucan synthase makes up the family (Coutinho *et al*. 2003).

1.5 Partial purification of β-1,3-glucan synthases and identification of catalytic subunits

In plants, callose (β-1,3-glucan) and cellulose (β-1,4-glucan) are produced by an enzyme complex which is capable of synthesizing both polysaccharides under tight regulation (Delmer, 1999). The callose synthase genes, specifically the catalytic subunits, have been studied in various plants including carrot (*Daucus carota L)* (Lawson *et al*., 1989), red beet *(Beta vulgaris)* (Frost *et al*., 1990), barley (*Hordeum vulgare)* (Pedersen *et al*, 1993), sunflower (*Lolium multiflorum)* (Bulone *et al*., 1990), cotton *(Gossypium hirsutum)* (Delmer *et al*., 1991; Li *et al*., 1993; Li and Brown, 1993), soy bean *(Glycine max)* (Fink *et al*,1991) and pea *(Pisium sativa)* (Dhugga and Ray, 1994; 1998). Callose is deposited in localized periplasmic spaces after wounding, stress or pathogen attack where the accumulated β-1,3-glucan is broken down again when conditions return to normal and the specific stress is relieved through a rapid regulated turnover process (Currier, 1957; Eschrich, 1975; Fincher and Stone, 1981; Mueller and Maclachlan, 1983; Waterkeyn, 1981). Callose synthase and cellulose synthase are part of an ancient gene family, containing signature domains and conserved residues. A translated amino acid sequence could function as a synthase for both β-1,3- and β-1,4-glucan synthesis possibly modulated by changes in enzyme phosphorylation states, Ca^{2+} or possibly through a modifiable peptide subunit (Hill and Brew, 1975). β-1,3-glucan synthase activity in pea, soybean (Kauss and Jeblick, 1985; Krauss and Jeblick, 1986; Kauss *et al.* 1983) and cotton membranes (Delmer *et al*., 1977; Delmer, 1983; Delmer *et al*., 1985) is increased significantly in the presence of Ca^{2+}. The mechanism of Ca^{2+} activation is unknown, although it appears to be specific for β-1, 3-glucan synthases and not by Ca^{2+} activated kinases or calmodulin-mediated activation (Girard and Maclachlan, 1987). Callose is specifically activated by Ca^{2+}, however several chemical and physical treatments also induce callose formation. Physical damage to plant tissue may increase Ca^{2+} concentrations at the membranes or alternatively, leaked endogenous proteases may activate zymogen-like β-1, 3-glucan synthase activity. In plants it seems possible that either or both protease and Ca^{2+} may regulate callose formation (Girard and Maclachlan, 1987). This may also explain the results were active UDP-glucose binding protein subunits are often isolated together with slightly larger molecular weight inactive proteins (Girard and Maclachlan, 1987).

In fungi, β-1,3-glucans are integral parts of the cell wall (Baskin *et al*., 1992) where some components exist as zymogens, where activity is regulated by proteolysis (Auer *et al*., 1998; Carpita *et al*., 1996). Genes encoding β-1,3-glucan synthases in fungi have been identified, sharing some identity with plant cellulose synthases. A gene encoding a large subunit of 200-230 kDa has also been identified which does not share

identity with plant or bacterial β-1,3-glucan synthases (Kelly *et al*., 1996). A 170 kDa polypeptide was identified in mung bean (Shin and Brown, 1998), which showed some identity with the large fungal β-1,3-glucan synthase gene. Similarly a tobacco pollen tube β-1,3-glucan synthase showed sequence similarity and related molecular weight to the fungal gene product (Turner *et al*., 1998). Amongst other examples, it seems likely that there is at least to some extent, some similarity between fungal and plant β-1,3-glucan synthases. The bacterial curdlan synthase gene encoding for a β-1,3-glucan synthase, was isolated and showed sequence similarities to cellulose synthase genes of bacteria and cellulose synthase genes of plants (Stasionopoulus *et al*., 1999). This suggests that the cellulose synthase genes of plants may also be responsible for β-1,3-glucan synthesis with slight modifications in the catalytic site amino acid sequence (Campbell *et al*., 1997; Legault *et al*., 1995).

Fungal cell walls are complex structures consisting of chitins, mannans, β-1,3-glucans and β-1,6- glucans. Several other fungal β-1,3-glucan synthase related gene products have been identified although not catalytic subunits, are thought to be involved in cell wall biogenesis (Enderlin and Selitrennikoff, 1994; Hong *et al*., 1994; Kasahara *et al*.,1994a; Roemer and Bussey, 1991; Roemer *et al*., 1993; Valdivieso *et al*., 1991). Modification of the regulatory GTPase subunit of *Saccharomyces cerevisiae*, Rho1p, is essential for β-1,3-glucan synthesis. Prenylation of the protein subunit by the geranylgeranyl transferase is required for activation of the β-1,3-glucan synthase catalytic subunit (Arellano *et al*., 1996; Diaz *et al*., 1993; Inoue *et al*., 1996; Inoue *et al*., 1999; Kondoh *et al*., 1997; Mazur and Baginsky, 1996; Quadota *et al*., 1996) while the geranylgeranyl transferase in turn is regulated by cytosolic Ca^{2+} levels (Inoue *et al*., 1999). KRE6 was reported to be the putative β-1,6-glucan synthase (Roemer and Bussey, 1991; Roemer *et al*., 1993) which is closely inter-linked with the β-1,3-glucan synthase (Shematek and Cabib, 1980).

Several methods of extraction, purification and identification have been attempted to isolate β-1,3-glucan synthases (Table 1). The most popular methods, specifically for callose synthases, has been to partially purify the protein subunits usually through product entrapment and further identifying substrate binding subunits through photo affinity labeling assays or immunoprecipitation. Detergents such as CHAPS, W1 and digitonin have been used to solubilize the membrane bound proteins although loss of activity and instability after solubilization has made detection based on enzyme activity difficult. Attempts to purify the enzyme in fungi have also been relatively unsuccessful (Aswald *et al*., 1993, 1994; Beauvais *et al*., 1993) again having problems with enzyme stability during solubilization although *Saccharomyces cerevisiae* β-1,3-glucan synthase was partially purified through CHAPS solubilization and product entrapment (Inoue *et al*.,

Table 1: Methods of purification and identification of β-1,3-glucan synthase proteins in literature.

Source of β-1,3-glucan	Methods of purification and identification	Detergent	Catalytic subunits	Non-catalytic proteins	References
Plant					
Beta vulgaris (Red Beet)	photo affinity labeling UDP-glycosyl transferase activity	digitonin	57 kDa		Frost *et al*., 1990
Daucus carota (Carrot)	photo affinity labeling UDP-glycosyl transferase activity	CHAPS	57 kDa	150 and 43 kDa	Lawson *et al*., 1988
Gossypium hirsutum (Cotton)	photo affinity labeling	digitonin	52 kDa	44 kDa	Delmer *et al*., 1991
	product entrapment photo affinity labeling	digitonin	37 kDa	32 and 66 kDa	Li *et al*., 1993
Hordeum vulgare (Barley)	product entrapment sucrose density gradient centrifugation UDP-glycosyl transferase activity	digitonin	36 and 52 kDa	54, 60, 70 and 94 kDa	Pedersen *et al*., 1993
Pisium sativa (Pea)	glycerol gradient centrifugation	digitonin	55 kDa		Dhugga and Ray, 1994; 1998
Glycine max (Soya bean)	polyethyleneglycol/dextran two phase separation sucrose density gradient centrifugation antibody affinity	digitonin	31 kDa		Fink, 1991
Phaseolus vulgaris (mung bean)	UDP-glycosyl transferase activity	digitonin	38 and 78 kDa	32, 54, 64 kDa	Kudlicka and Brown, 1997
Lolium multiflorum (Sunflower)	Immunoprecipitation photo affinity labeling	CHAPS	31 kDa	30, 54 and 58 kDa	Meikle *et al*., 1991
Bacterial					
Agrobacterium tumefaciens	Functional complementation		59 kDa		Stasinopoulos *et al*., 1999
Fungal					
Saproglenia monoica	Product entrapment UDP-glycosyl transferase activity assay	CHAPS	34, 48 and 50 kDa		Bulone *et al*., 1990
Saccharomyces cerevisiae	Product entrapment Immunoprecipitation	CHAPS	200 kDa		Inoue *et al*., 1995
Cordyceps militaris	Cloned using sequence similarity		1981 aa		Ujita *et al*., 2006
Aspergillus fumigatus	Cloned using sequence similarity	CHAPS	218 kDa	21.5, 100 and 160 kDa	Beauvais *et al*., 2001
Neurospora crassa	Functional complementation			59 kDa	Enderlin and Selitrennikoff, 1994

1995). Functional complementation screening methods have been used more recently to identify β-1,3-glucan synthases (Enderlin and Selitrennikoff, 1994; Stasinopoulos *et al*., 1999).

1.6 Paramylon synthase

Bäumer *et al*., (2001) partially purified β-1,3-glycosyl transferase proteins from *E. gracilis* through separation by sucrose density centrifugation of proteins purified from the paramylon granular membrane through CHAPS solubilization. A 670 kDa enzyme complex was separated with UDP-glycosyltransferase activity, a molecular weight larger than that of most of the β-1,3-glycosyltransferase complexes in plant and bacterial cell membranes (Kudlicka and Brown., 1997; Li and Brown., 1993; Thelen and Delmer, 1986).

Bäumer *et al*. (2001) reported that paramylon synthesis is optimal with Ca^{2+}, spermidine (0.6 mM), CHAPS (0.15%) and a paramylon primer, similar to the requirements for cotton, carrot and green bean β-1,3-glucan synthases which require Ca^{2+} and a detergent, either digitonin or CHAPS (Hayashi *et al*., 1987; Lawson *et al*., 1989; McCormack *et al*., 1997). Cytosolic Ca^{2+} concentrations over 200 nM signal tissue damage in higher plants triggering a wound repair response where callose β-1,3-glucan is synthesized while normal conditions (200 nm Ca^{2+}) induce cellulose β-1,4-glucan synthesis (Li *et al*., 1993). In *E. gracilis* however no non specific glucan polymers were synthesized, only paramylon synthesis activity was affected by variations of Ca^{2+} (Bäumer *et al*., 2001).

Bäumer *et al*. (2001) concluded that the paramylon synthase complex had a relatively high molecular weight (~670 kDa) with protein subunits similar and within range of those in previous studies (31 to 83 kDa) (Kudlicka and Brown., 1997) which could bind UDP-glucose as substrate under the positive influence of effectors.

1.7 Cytokine–related immunopotentiating activities of paramylon

β-1,3-glucans have drawn interest in the pharmacological field as biological moderators in human and animal systems specifically as immuno-potentiators, anti-inflammatory, antimicrobial, antitumor, hepatoprotective, antifibrotic, antidiabetic, hypoglycemic and anitcholesterol activities (Brown and Gordon, 2003; Kojima *et al*., 1986; Robbins and Seely, 1977; Sutherland, 1998; Trepel, 2004).

The macromolecular structures of the β-1,3-glucans are dependent on the sources of the glucans as well as the isolation procedure. *Euglena gracilis* paramylon is a water insoluble linear triple helical β-1,3–glucan (122 kDa), shown to have structural similarities to strong immunopotentiating β-1,3–glucans such as lentinan and schizoflan (Marchessault and Deslandes, 1979). Antitumor activity of native paramylon was reported to be relatively weaker than other pharmacologically active β-1,3-glucans, where only partial tumor regression was observed and most effectively at lower doses (1mg/kg/day x10) rather than higher doses (5mg/kg/day x10) (Quesada *et al*., 1976). It has been suggested that paramylon could have greater potential due to its strong adjuvant activity other than antitumor responses, more specifically for the activities of paramylon on humoral antibody and cytokine production. The immunopotentiating effects of paramylon as an adjuvant has been observed at much higher doses (10 and 50 mg/kg) indicating that the adjuvant and antitumor activities follow different mechanisms (Kondo *et al*., 1992). Paramylon administered to mice caused an increase in the levels of cytokine IL-6, which caused an differentiation of activated B cells to matured B cells (Hirano *et al*., 1985; Muraguchi *et al*., 1988), and high amounts of IL-1, which together with IL-6 caused an increase in T cell activation (Marchessault and Deslandes, 1979), as well as the induction of hematopoeisis for early precursor cells in bone marrow (Ikebuchi *et al*, 1987; Wong *et al*., 1988). The increase in IL-1, which appears higher than that of lentinan and schizoflan, have been shown to have multiple activities including T cell activation as well as IL-2 and IL-4 production and expression of IL-2 receptors (Ho *et al*., 1987; Lowenthal *et al*., 1980; Smith *et al*., 1980). Paramylon appears to have greatest immunomodulating activities at higher doses and specifically on cytokine production. Anti-oxidative mechanisms were enhanced in the liver after paramylon administration, significantly reducing acute hepatic injury (Sugiyama *et al*., 2009).

Several biologically active, specifically antimicrobial active derivatives of paramylon have been synthesized, positively charged derivatives including N,N-dimethylaminoethyl (DMAE), N,N-diethylaminoethyl (DEAE) and 2-hydroxy-3-trimethylammoniopropyl (HAP) were effective at increasing macrophage activities, specifically increasing NBT-reducing activities compared to negatively charged and neutral derivatives. However, carboxymethyl paramylon and paramylon sulfate did not significantly enhance specific macrophage antimicrobial activities (Sakagami *et al*., 1989; 1991), only paramylon sulfate has effective anti-HIV activities against HIV-1nTLVmm, HIV-1AO12B and HIV-2ROD in PBMC and MT-4 cells (Baba *et al* 1988; De Clercq, 1986; Ito *et al*. 1987; Mizumoto *et al*, 1988; Nakashima *et al*, 1987;Ueno and Kuno, 1987). The anti-HIV activities of paramylon sulfate was completely dependent on molecular weight and degree of sulfation (Baba *et al*, 1990; Daniel *et al*, 1985; Itoh *et al*, 1990; Witvrouw *et al*, 1991), where paramylon sulfate completely inhibited HIV binding to cells in a dosage range compatible with *in vivo* administration

(Koizumi *et al*, 1993). Although paramylon sulfate had a lower inhibition of HIV binding compared to dextran sulfate (Baba *et al*, 1988; Mitsuya *et al*., 1988; Nakashima *et al*., 1989; Schols *et al*., 1989), it was still higher than the inhibition of HIV binding by polyphenolic compounds (Manabe *et al*, 1992; Nakashima *et al*, 1992a; 1992b). The synergistic anti-HIV effect was increased when combinations of azidothymidine (AZT) and paramylon sulfate were optimized, reducing therapeutic dose requirements and anti-HIV activity (Koizumi *et al*., 1993). Antiviral activities are only specific for HIV (Fukuchi *et al*., 1989; Harada *et al*., 1991)

1.8 Aims and experimental approach

The aim of this study was to identify genes involved in the synthesis of paramylon, specifically the catalytic β-1,3-glycosyl transferase genes. In other studies, several methods have been used in attempt to isolate and characterize the paramylon synthase genes including functional screening of cDNA libraries, where various detection methods were used (van der Merwe, 2007) as well as protein purification approaches (Bäumer *et al*., 2001; van der Merwe, 2007). In this study, several strategies have been followed, one of the strategies has been to construct and screen a cDNA library using a wider range of screens than in previous studies.

A *Euglena gracilis* cDNA lambda phage library was constructed which expressed gene products by lysing XL1- blue *E. coli* or expressing the cDNA library inserted into pBluescript plasmids in *E. coli* colonies. Aniline blue served as a functional screen as it specifically stains β-1,3-glucans although this strategy relies on the proteins being actively expressed in *E. coli*, as well as the stain being active within the bacterial colonies. Another technique was to use the ZAP cDNA synthesis kit in combination with the Pico Blue immunoscreening protocol where the phage library was expressed in the form of Lambda phage containing pBluescript vectors containing *E. gracilis* cDNA inserts. Lysis of bacterial lawns by the phage library would express the cDNA gene products. The gene products would be expressed and blotted onto a nitrocellulose membrane and blocked. These membranes would be screened either using radio-labeled substrate in the form of [^{14}C] UDP-glucose where the substrate binding protein subunits (Bäumer *et al*., 2001; van der Merwe, 2007) would be screened. Another approach was to screen phage libraries with an antibody raised against the proteins associated to the paramylon granule as this would not be dependent on functional activity for detection. The cDNA library was amplified and purified by mass excision in the form of pBluescript. This allowed for expression of the gene products directly in *E. coli* systems where they were screened by lysing the bacterial colonies and detecting the gene products using the antibody. The mass

excision was also screened using primers to amplify DNA based on consensus sequences derived from β-1,3-glucan synthases from several organisms as well as degenerate and non degenerate primers designed from a peptide sequence isolated from purified paramylon synthase proteins.

Functional and structural instability of the isolated proteins have caused difficulty in initial attempts to purify these proteins in other systems (Delmer, 1999). However, a relative degree of success has lead to isolation of catalytic subunits through protein purification in conjunction with the available sequence information, where peptide sequences isolated were compared to pre-existing sequenced genomes. Due to the relative lack of sequence information available for *E. gracilis*, protein purification methods to deduce gene information rely greatly on a degree of identity to already characterized reference genes.

The protein purification strategy adopted for this study aimed at purifying proteins associated with the paramylon granule membrane. Previous studies were partially successful at partially purifying two peptide subunits with substrate affinity comparable to those found in plants (Bäumer *et al*., 2001; van der Merwe, 2007). This study has followed on from previous methods in order to partially purify and derive sequence information from peptides associated with the granule membrane.

A transcriptomic approach was also followed to isolate transcript sequence information from *E. gracilis*. As the genome is made up of a chimeric collection of genes inherited from a combination of a heterotrophic kinetoplastid and the chloroplast of a photosynthetic eukaryote obtained during a secondary endosymbiotic event, making *E. gracilis* taxonomically complex with respect to both morphology and genomics as no comparable reference genome exists. The chloroplast of *E. gracilis* has already been sequenced along with several ribosomal and EST sequencing projects already (Ahmadinejad *et al*. 2007) in order to gather sequence information specifically for taxonomic and expression profile studies. During this study, an un-annotated genome was sequenced and released by another group in a taxonomic study, which was then also used in this study.

In this study, the transcriptome was sequenced under conditions optimal for paramylon synthesis using the genome sequencer FLX-system specifically to identify genes involved in paramylon synthesis. Previous attempts to sequence *E. gracilis* ESTs have not identified any β-1,3-glycosyl transferases although short glycosyl transferases GT-2 sequences were identified on Genbank as well as β-1,3-glycosyl hydrolases.

Materials and Methods

2. Materials

2.1 Chemicals and kits

Chemicals and kits used were obtained from Sigma (St. Louis Missouri, USA), Roche Diagnostics (Mannheim, Germany), Promega (Madison, Wisconsin, USA), Stratagene (La Jolla, California, USA), Invitrogen (Carlsbad, California, USA), CalBiochem (Merck Biosciences, Darmstadt, Germany), BioRad Laboratories GmbH (Munich, Germany), Fermentas, Amersham biosciences (Uppsala, Sweden) GE healthcare (Uppsala, Sweden).

Paramylon synthase specific primary antibodies were produced by the Department of Biochemistry, University of Stellenbosch, South Africa. Secondary antibodies were obtained from Sigma (St. Louis Missouri, USA).

Euglena gracilis Z Krebs strain SAG 1224-5/25 obtained from Algensammlung, Göttingen, Germany.

SOLR™ strain (Stratagene) e14^{-} (McrA^{-}) Δ(mcrCB-hsdSMR-mrr) 171 sbcC recB recJ uvrC umuC::Tn5 (Kan^{r}) lacgyr A96 relA1 thi-1 endA1 λ^{R} [F´ proAB lac^{q}ZΔM15] Su^{-} (nonsuppressing)

XL1- Blue MRF´ strain (Stratagene) Δ(McrA) 183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F´ proAB lac^{q}ZΔM15 Tn10 (TET^{r})]

DH5α strain (Promega): F'/end*A1 hsdR17*($r_k^-m_k^+$) *supE44 thi-1 recA1 gyrA* (Na^{r}) *relA1 Δ*(*lacZYA-argF*) deoR (Φ80d*lacΔ*(*lacZ*)M15)

BL21 strain (Invitrogen)*: E. coli* B F^{-}, ompT*, hsdS (r_B^-, m_B^-), gal, dcm.*

2.2 Primers

All primers were obtained from IDT (USA) and were designed using NCBI primer design. Primers were designed based on β-1,3-glucan synthase sequences (Table 2) which were aligned using EBI/t-coffee where multiple alignments and conserved domains were identified. Forward and reverse degenerate primers were designed based on conserved amino acid sequences (Table 3) according to the codon

usage efficiency of *E. gracilis* (Nakamura *et al*., 2000). The number assigned to the primers are based on the location of the sequence relative to the *Candida albicans* β-1,3-glucan synthase.

Table 2: Conserved β-1,3-glucan synthase sequences used to design primers.

Source of β -1,3-glucan synthase	**NCBI Accession number**
Candida albicans	ref|XP_721429.1|
Lolium multiflorum	gb|AAQ17229.1|
Schizosaccharomyces pombe	ref|NP_594766.1|
Saccharomyces cerevisiae	gb|AAR86936.1|
Scedosporium prolificans	gb|ABY53595.1|
Arabidopsis thaliana	ref|NP_567278.1|

Table 3: Degenerate primers designed from β-1,3-glucan synthase conserved domains.

Primer CD1	**1075-1125**
Fw:	5'- AAC CAG GAY AAC YAC HTI GAG GAG -3'
Rev:	5'- CTC CTC NAI GTR GTT RTC CTG GTT -3'
Primer CD2	**943-963**
Fw:	5'- TGG GCN AGC TWS CGS KSY CAG CAN YTS -3'
Rev:	5'- SAR NGT CTG RSM SCG SWA GCT NGC CCA -3'
Primer CD3	**1191-1204**
Fw:	5'- HTN CAC TAC GGN CAC CCN GAY -3'
Rev:	5'- RTC NGG GTG NCC GTA GTG NAI -3'
Primer CD4	**1210-1232**
Fw:	5'- GAG GAY ATY TYC GCA GGA YTS -3'
Rev:	5'- SAR TCC TGC GRA RAT RTC CTC -3'
Primer CD5	**1421-1437**
Fw	5'- GAG TGC YTY TGC TWC ATW TWC MAC -3'
Rev	5'- GTK GWA WAT GWA GCA RAR GCA CTC -3'
Primer CD6	**321-332**
Fw	5'- SCC GAG TGC CTG TGC TWC ATY -3'
Primer CD7	**392-400**
Fw	5'- TAC GAY GAY IKY AAC SAG HWS TTC TGG -3'
Rev	5'- CCA GAA SWD CTS GTT RMI TTC RTC GTA -3'

2.3 Primers designed from an isolated *E. gracilis* β-glucan synthase sequence

A short peptide sequence (KMCFTQGYLEFSARL) was predicted from a previous study from a ~50 kDa protein subunit purified and solublilized from the paramylon granular membrane through SDS-PAGE purification, tryptic digestion and LCMSMS (van der Merwe, 2007). The peptide sequence showed complete homology to a *KRE6* β-1,6-glucan synthases from *Debaromyces hanseii* and partial homologyidentity to *KRE6* β-1,6-glucan synthases from *Saccharomyces cerevisiae* and *Candida albicans*. Forward and reverse primers were designed (Table 4) based on the predicted sequence using codon usage efficiency of *E. gracilis* (Nakamura *et al*., 2000).

The *Debaromyces hanseii SKN1* (XP 002770128.1), *Candida tropicalis KRE6* (XP 002547982.1) and *SKN1* (XP 002547983.1) β-1,6-glucan synthases have conserved WN amino acids on the N-terminal of the KMCFTQGYLEFSARL sequence of the glucan synthase peptide which were included in the primer design as they reduced degeneracy of the primers.

Table 4: Primers based on the *D. hanseii* glucan synthase peptide**.**

Primer	**β-glucan synthase primer (MCFTQGY)**
Fw non deg	5'- ATG TGC TTT ACC CAA GGG TAC T -3'
FW deg	5'- CG ATG TGC TTY CAN CAR GG -3'
Primer	**β-glucan synthase primer (WNKMCFT)**
Fw non deg	5'- TGG CAG AAG ATG TGC TTC AC -3'
FW deg	5'- TGG CAR AAR ATG TGY TTY AC -3'

2.4 Web – based programs

Table 5: Website-based bioinformatics tools.

Database/ Program	**http site**	**Reference**
Bioedit	http://www.mbio.ncsu.edu/BioEdit/	Hall, 1999
NCBI BLAST	www.ncbi.nml.nih.gov/Blast/	Altschul *et al*., 1990
CLUSTALW	www2.ebi.ac.uk/Clustalw	Thompson *et al*., 1994
Euglena EST Database	tbestdb.bcm.umontreal.ca/	O'Brien *et al*., 2007
EBI/ t-coffee	http://www.ebi.ac.uk/Tools/t-coffee/	Notredame *et al.,* 2000
Codon usage table	http://www.kazusa.or.jp/codon	Nakamura *et al*., 1997
Mascot	www.matrixscience.com/	Hirosawa *et al*., 1993
GeneMark	http://exon.biology.gatech.edu/	Besemer and Borodovsky, 1999
CLC genomics workbench	http://www.clcbio.com/	Droege and Hill, 2008

3. Methods

3.1.1 Construction and screening of the cDNA library

3.1.1.1 Growth conditions of *Euglena gracilis*

Euglena gracilis was grown under heterotrophic conditions, at 22˚C, on media consisting of 10g tryptone, 4g yeast extract and 30g D-glucose per L. Liquid cultures were grown on a rotational shaker at 130 rpm and additional glucose was supplemented to 2% (m/v) once a week. Solid media contained 1.5% agar and were transferred to new plates every month.

3.1.1.2 Total RNA extraction

RNA was extracted from liquid cultures. Cells were centrifuged at 4,200g for 10min. Cells were homogenized in liquid nitrogen and added to 5 volumes extraction buffer preheated at 65˚C consisting of 2% CTAB, 2% PVP, 100mM Tris-HCl pH 8.0, 25mM EDTA, 2M NaCl, 0.05% spermadine trihydrochloride and 3% β–mercaptoethanol. Extracts were vortexed immediately and incubated at 65˚C for 5 min. After incubation the extracts were centrifuged at 8000g and the supernatant was transferred to a new tube. One volume of chloroform: isoamyl alcohol (24:1) was added to the supernatant, vortexed for 30 seconds and centrifuged again at 6000g for 15min. The aqueous layer was pipetted into a new tube and this step was repeated. LiCl was added to a final concentration of 2M and the RNA was precipitated overnight at 4˚C. The precipitated RNA pellet was collected by centrifugation at 10000g for 60min and washed in 500ul 70% ethanol. The washed RNA was centrifuged at 10000g for 15min and dried. The RNA was resuspended in distilled water.

3.1.1.3 Isolation and quantification of mRNA

Messenger RNA was purified from total RNA using the Genelute™ mRNA miniprep kit (Sigma MRN10KT 0461K6879) according to product guidelines. All RNA was quantified using a nanodrop 2000 (Thermo Fischer Scientific Inc., USA). Concentrations were measured at 260nm and RNA quality was calculated as the ratio between 260 and 280nm and samples were tested using 1% agarose gel electrophoresis.

3.1.1.4 cDNA synthesis

First strand cDNA synthesis from isolated mRNA was performed using the Revertaid™ H Minus First strand cDNA synthesis Kit (Fermentas) according to product guidelines. Second strand synthesis of cDNA was performed using the second strand cDNA synthesis kit (Stratagene, #200401-5) according to the product guidelines. Purified second strand cDNA was quantified using a nanodrop 2000 (Thermo Fischer Scientific Inc., USA). The quality of the cDNA was determined by agarose gel electrophoresis. The purified second strand cDNA was quantified using a nanodrop 2000 (Thermo Fischer Scientific Inc., USA). The quality of the cDNA was determined by agarose gel electrophoresis.

3.1.1.5 cDNA library construction and in vitro packaging of the phage library

The cDNA library was constructed using the Zap cDNA Synthesis kit (Stratagene), *E. gracilis* total RNA was extracted and Poly(A)$^+$ RNA was purified using the Genelute™ mRNA miniprep kit (Sigma). First strand cDNA synthesis, second strand cDNA synthesis, size fractionation, ligation of the cDNA insert into the Uni-Zap XR vector and all related steps were performed according to the product guidelines. The cDNA Uni- Zap XR ligated vector was packaged using the Gigapack III Gold cloning kit (Stratagene) according to the product guidelines.

3.1.1.6 Amplification of the cDNA primary library

XL1-Blue MRF´ cells (Stratagene) were used for tittering and the construction of the amplified library following the instructions of the Gigapack III Gold cloning kit (Stratagene) manual. The titer was calculated (186 plaques per plate 1:10 dilution). The amplified library was collected and stored in SM buffer (100mM NaCl, 50mM Tris-HCL pH. 7.5, 10mM $MgSO_4$) in 2ml aliquots at -80°C.

3.1.1.7 Mass excision of the amplified library

A mass excision of the amplified library was performed using XL1-Blue MRF´ (Stratagene) and SOLR™ (Stratagene) bacterial strains and the ExAssist helper phage according to the Gigapack III Gold cloning kit (Stratagene) manual. The mass excision was performed according to the titer of the amplified library. Following the mass excision the phagemid library was purified using the Nucleobond® AX nucleic acid purification kit (Macherey-Nagel, Germany).

3.1.1.8 Single clone excision

Plaques of interest were cored out from agar plates. And transferred to new tubes and stored in SM buffer (100mM NaCl, 50MM Tris-HCl pH 7.5 and 10mM $MgSO_4$) and 20µl chloroform. Phages were extracted and the pBluescript phagemid vectors containing ligated inserts were purified according to the Gigapack III Gold cloning kit (Stratagene) guidelines. Phagemid vectors were transformed into SOLR™ (Stratagene) bacterial cells. Plasmid DNA was prepared from colonies and the plasmids were sequenced.

3.1.2 Screening of the phage library

3.1.2.1 Infection and Expression of the phage library

The phage library was screened using the PicoBlue™ Immunoscreening Kit (Stratagene) according to the manufacturer's guidelines. Cell cultures of XL1-Blue MRF´ (Stratagene) were grown overnight in NZY broth (5g NaCl, 2g $MgSO_4$,.$7H_2O$, 5g yeast extract, 10g NZ amine supplemented with 2% maltose) and resuspended in 10mM $MgSO_4$ and 2% maltose to an OD_{600} of 0.5. Known titers of Lambda Zap phage from the amplified library were used to infect the 200µl aliquots of cell cultures. The infected cell cultures were incubated at 37˚C for 15min and added to NZY top medium (0.7% agarose) pre warmed to 48˚C and poured onto pre warmed NZY plates. The plates were incubated upside down for three and a half hours at 37˚C until small phages appeared. Nitrocellulose membranes (Millipore), pre soaked and 10mM IPTG and dried, were placed on the lysed bacterial lawns and the positions of the membranes were marked using a sterile needle. The plates were incubated with the membranes for another four hours at 37˚ and then briefly at 4˚C. The membranes were lifted for further screening and the plates were stored at 4˚C.

3.1.2.2 Screening of the phage library using an antibody

The membranes were screened using a polyclonal primary antibody raised in rabbits (University of Stellenbosch, South Africa). The antibody was raised against proteins associated (bound) to the paramylon granule.

Membranes lifted from phage infected plates were washed in TBST (20mM Tris-HCl pH 7.5, 150mM NaCl and 0.05% Tween 20) and blocked in blocking solution consisting of TBST and 1% BSA (Bovine Albumen (Fraction V), Roche) for two hours. Membranes were washed and blocked in square containers with several membranes per container and 8ml/membrane blocking solution. The primary antibody was added to fresh blocking buffer in a 1:500 dilution and incubated overnight. The membranes were washed in TBST 5 times and incubated for one hour in TBST with the secondary goat anti rabbit – alkaline phosphatase antibody in a 1:20000 dilution. The membranes were washed five times in TBST and once in TBS (TBST without Tween 20) and incubated in a solution of NBT/BCIP Ready-to-use tablets (Roche) until colour development was observed. The membranes were washed extensively with water to stop the colour development reaction.

The excised phages were vortexed and centrifuged at 10000g for 5 min. The phages of interest were screened again by infecting fresh XL1-Blue MRF´ (Stratagene) cells, repeating the screening process for secondary and tertiary screens using 2 µl of the isolated plaque for infection. Phages that appeared to produce signal during secondary and tertiary screens were extracted by single clone excision.

3.1.2.3 Screening of the phage library using radio-labeled substrate

Nitrocellulose membranes (Sigma-Aldrich) were screened using [^{14}C] UDP-glucose. Membranes lifted from phage infected plates were washed in TBST and blocked in buffer in square containers containing approximately 8ml buffer per membrane. Membranes were blocked in 4% BSA-TBST solution for two hours. The membranes were incubated in 250mM Tris-HCl pH 7.5 and 10µl [^{14}C] UDP-glucose for 1 hour at room temperature with gentle agitation. The membranes were dried and exposed to a Super resolution Cyclone Phosphor screen (Packard). After seven days the hybridization was detected with a Cyclone TM Storage Phosphor system phosphor imager (Packard instrument Co., Meriden, USA). Radioactive signals which corresponded to phages were determined and the phages were excised and stored in 500µl SM buffer (100mM NaCl, 50MM Tris-HCl pH 7.5 and 10mM $MgSO_4$) and 20µl chloroform.

The excised phages were vortexed and centrifuged at 10000g for 5 min. The phages of interest were screened again by infecting fresh XL1-Blue MRF´ (Stratagene) cells, repeating the screening process for secondary and tertiary screens using 2 µl of the isolated plaque for infection. Phages that appeared to produce signal during secondary and tertiary screens were extracted by single clone excision.

3.1.2.4 Functional screening of the mass excision library using aniline blue

The mass excised pBluescript vector based phage library was transformed into Bl21 *E. coli* competent cells. Approximately 100ng DNA of the plasmid library was used to transform 1 ml of Bl21 competent cells per large plate made up of LB solid medium with ampicilin and 2 mM IPTG added. The transformed cells were added to 10ml/ plate LB medium containing 0.7% agarose and ampicilin and poured onto the plates. The embedded transformed colonies were misted using a spray bottle with aniline blue solution (0.0025% aniline blue (Sigma) dissolved in 0.1M K_3PO_4 pH 12) and incubated open at room temperature for 30 min on a laminar flow bench. The plates were washed with 1 M NaCl to destain the colonies. The plates were viewed under ultraviolet light to identify possible positive colonies staining yellow.

3.1.2.5 Screening of the mass excision library using cell lysis

The immunological screening of the phage expression library was performed with transformed bacterial colonies according to the Sambrook and Russel, 2001. Nitrocellulose membranes (SIGMA, Millipore #N-7395) were placed on LB solid medium containing ampicilin. A culture of SOLAR cells (Stratagene) were inoculated from a single colony and grown in LB liquid media containing ampicilin until the OD_{600} reached 0.5. A tittered amount of phage was used to inoculate the cell culture and incubated at 37˚C for 15 min. The phage inoculated cell cultures were aliquoted onto the nitrocellulose membranes and spread evenly using a sterile glass rod. The plates were incubated for approximately 12 hours at 37˚C until small bacterial colonies appeared. Replicate nitrocellulose membranes were placed on top of the primary membranes and the orientations were marked using a sterile needle. The replicate membranes were placed onto new LB plates containing ampicilin and 2 mM IPTG (isopropylthio-β-D- thiogalactopyranoside) and incubated for approximately 8 hours until the replicate bacterial colonies formed. The colonies on the nitrocellulose membranes were then fixed by exposing the membranes to chloroform fumes for 20min in a closed container. The bacterial colonies on the membranes were then lysed by incubating the membranes for 16 hours at room temperature on a shaker in blocking solution (TBST containing 4% BSA) with 1mg/ml 0.1% lysozyme and 0.05% [w/v] sodium azide added. Membranes were washed and blocked in large round Petri dishes with two membranes per container and 10 ml/membrane solution. The primary antibody was added to fresh blocking buffer in a 1:500 dilution and incubated overnight. The membranes were washed in TBST 5 times and incubated for one hour in TBST with the secondary goat anti rabbit – alkaline phosphatase antibody in a 1:20000 dilution. The membranes were washed five times in TBST

and once in TBS (TBST without Tween 20) and incubated in a solution of NBT/BCIP Ready-to-use tablets (Roche) until colour development was observed. The membranes were washed extensively with water to stop the colour development reaction.

3.1.3 cDNA synthesis for PCR screening and molecular cloning

3.1.3.1 Design of PCR primers

Primers were designed (Section 2.4) from short peptides sequences isolated from paramylon synthase peptides purified from *E. gracilis* (van der Merwe, 2007). Degenerate and non degenerate primers were designed and used to screen both the mass excision library as well as cDNA for sequence. Primers were also designed based on conserved domains of β-1,3-glycosyl transferase shared by a number of organisms.

Degenerate and non-degenerate primers were designed based on a peptide sequence isolated from a peptide partially purified from *E. gracilis* showing UDP-glucose binding affinity which separated at approximately 37 kDa on SDS PAGE. The peptide was digested with trypsin and the sequence was obtained by LCMS (van der Merwe, 2007). These primers were used as well as new primers designed from the same sequence for this study.

3.1.3.2 PCR conditions

PCR conditions varied for Tm and elongation times depending on the primer pairs used. PCR conditions for cDNA templates were denaturation at 94°C for 2 min, 35 amplification cycles of 94°C for 45s, 30s at 55 or 60°C and 60s or 90s at 72°C and final elongation for 7min at 72°C. PCR conditions for mass excision plasmid templates varied for Tm and elongation times. As pBluescript formed the vector backbone of the mass excision library, the T3 primer (5' – ATTAACCCTCACTAAAGGGA – 3') was used as an anchored primer, as the direction of cDNA insertion was in this direction (Zap cDNA Synthesis kit guidelines, Stratagene), where the conserved domain reverse primers and designed peptide primers were paired with the T3 primer, where the T7 primer (5' – TAATACGACTCACTATAGGG – 3') was paired with the conserved domain forward primers. PCR conditions were 2 min denaturation at 94°C, 35 amplification cycles of 94°C for 30s, 55°C for 30s and 72°C for 90s and final elongation at 72°C for 7min.

3.1.3.3 DNA purification, cloning, transformation and sequencing

PCR products were separated using agarose gel electrophoresis, stained with ethidium bromide and visualized using the Alpha imager. All PCR products were excised and purified using the QIAquick® Gel Extraction kit (QIAGEN) according to the product guide lines. Purified PCR products were ligated using the Clonejet™ PCR Cloning Kit (Fermentas) according to the product guidelines. All bacterial transformations were performed according to using the heat shock method (Maniatis protocol). Colonies that were selected were screened using the CloneJET™ kit's primer pair (forward sequencing primer: 5'-CGACTCACTATAGGGAGAGCGGC-3' and reverse sequencing primer 5'-AAGAACATCGATTTTCCATGGCAG-3'. Vectors containing inserts of the correct sizes were excised and sequenced using the T7 primer as described. All sequencing analysis was done at the Central Analytical Facility, University of Stellenbosch, using the ABI Prism 3730 Genetic Analyser using bigDye terminator cycle sequencing ready reaction.

3.2 Partial purification of paramylon synthase proteins

3.2.1.1 Protein extraction

Crude protein was extracted according to a modified method of Bäumer *et al.* (2001). Heterotrophic cell cultures were centrifuged at 4,200g for 10min in a Sorval centrifuge (Sorval RC5C Plus, Kendra Lab products, USA). The pellets were washed twice with ice cold distilled water and resuspended in buffer A (50mM Tris-HCl pH 7.4, 250mM sucrose, 3mM EDTA, 1mM dithiotreitol (DTT, Roche) and 1 tablet Complete™ protease inhibitor (Roche) per 50ml extraction buffer). Cells were disrupted by sonication using a virsonic 60 sonicator (The Viritis Co. Inc., Gardiner, New York, USA) at maximum for four 15 sec bursts and 30 seconds intervals on ice. The paramylon granules was centrifuged at 1500g for 5min at 4˚C, washed four times in buffer A and resuspended in buffer B (25mm Tris-HCl pH 7.4, 20% (w/v) sucrose and 1mM DTT). The protein was solubilized from the paramylon granules by adding a solution of the detergent CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate, Sigma) drop wise to a final concentration of 0.15% (w/v). The proteins associated to the paramylon granule were solubilized overnight with gentle stirring on ice. The paramylon granules were separated from the disassociated protein by centrifugation at 2000g for 5min at 4˚C. The partially purified protein extracts were stored at -20˚C.

3.2.1.2 Dialysis of the crude protein extract

Partially purified protein samples were desalted using 10,000 MW Slide-A-Lyzer® Dialysis Products (Pierce, #66810). Protein samples were loaded into the slide according to manufacturer's guidelines. The slide was desalted in 2l of an equal strength buffer (25 mM Tris-HCl pH (7.4) and 1mM DTT) overnight with gentle stirring until the protein sample became isotonic with the buffer.

3.2.1.3 Protein quantification

Protein was quantified according to the method of Bradford (1976) using the BioRad protein assay reagent (BioRad, #500-0006). The protein concentration was calibrated using a Bovine Serum Albumin (BSA) standard curve. Protein was quantified by measuring the absorbance at 595 nm using a µQuant microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, Vermont, USA). Protein samples partially purified with CHAPS were quantified using the BCA™ Protein Assay Kit-Reducing Agent Compatible (Pierce, Cat. 23250, Rockford Illinois, USA) according to the product guidelines. Absorbance was measured at 562 nm using a µQuant microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, Vermont, USA) calibrated against a BSA standard curve.

3.2.2 FPLC, PAGE separation, product entrapment and immunoblotting of partially purified paramylon synthase proteins

3.2.2.1 FPLC of paramylon synthase proteins

The anti-paramylon synthase antibody was purified using FPLC and coupled to activated CNBr-sepharose. Dialysed protein extract was purified by affinity binding FPLC of the antibody coupled to activate CNBr-sepharose.

Polyclonal antibody serum (500µl) was desalted using an Amicon Ultra 10 000 kDa MWCO centrifuge column and purified through the HiTRAP Protein A with sample size at 1ml, where 20mM sodium phosphate, pH 7.0 was used as the binding buffer where 0.1 M citric acid pH 4.5 was used for elution. The eluted purified polyclonal antibody was resuspended in CNBr-activated Sepharose 4B coupling buffer (0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl) and coupled to CNBr-activated Sepharose 4B (GE healthcare) for 2 hours at room temperature and washed with three cycles of 0.1 M acetic acid/

sodium acetate, pH 4.0 containing 0.5 M NaCl alternating with 0.1 M Tris-HCl, pH 8 containing 0.5 M NaCl. Dialyzed CHAPS solubilized protein samples (Section 3.2.1.2.) were equilibrated in the binding buffer and desalted using a PD-10 column (GE Healthcare).

Affinity binding of dialysed protein extract was performed using an ACTA prime FPLC with the HiTRAP Protein A sample size at 1ml, where 20mM sodium phosphate, pH 7.0 was used as the binding buffer where 0.1 M citric acid pH 4.5 was used for elution. Dialysed protein (2.5 ml, ~5000 mg) was injected into the antibody coupled sepharose column where proteins bound to the CNBr coupled antibodies in binding buffer (75mM Tris-HCl, pH 7.4) and then eluted with elution buffer (0.1 M glycine-HCl pH 2.7, 0.5 M NaCl).

3.2.2.2 Detection of in gel paramylon synthase activity

Partially purified native proteins disassociated from the paramylon granule were separated using native PAGE. An 8% resolving gel (4.7 ml H_2O 2.7 ml acrylamide:bis-acrylamide (30%:0.8%), 2.5 ml 1.5M Tris-HCl (pH 8.8), 0.1 ml 10% Ammonium persulfate and 8 µl TEMED made up to 10 ml) A 5% stacking gel (3.1 ml H_2O, 0.67 ml acrylamide:bis-acrylamide (30%:0.8%), 0.5 ml 1.0M Tris-HCl (pH 6.8), 0.04 ml 10% Ammonium persulfate and 4 µl TEMED made up to 4 ml). The running buffer consisted of 25mM Tris-HCl (pH 8.3) and 192mM glycine. The proteins were separated at 0˚C and following electrophoresis the gel was equilibrated for 1 hour in 25 mM sodium phosphate buffer (pH 7.4), 1mM $CaCl_2$ and incubated overnight in 25mM sodium phosphate buffer (pH 7.4), 2% [w/v] UDP-glucose, 1 mM $CaCl_2$ at room temperature. A lane from the gel was cut off and incubated separately overnight as a control in 25mM sodium phosphate buffer (pH 7.4) and 1 mM $CaCl_2$ at room temperature. The gels were fixed in a solution of 40% methanol 7% acetic acid for 30 min and oxidized in a solution of 1% periodic acid and 3% acetic acid for 60 min. The gel was washed in ddH_2O to remove the acid. The gel was incubated for 60 min in Schiff's stain (SIGMA-ALDRICH, #S5133-500ml) to visualize the glucan bands.

3.2.2.3 Partial purification of paramylon synthase through product entrapment

Partially purified proteins (approximately 2000 mg), solubilized with CHAPS from the paramylon granule, and were further purified by incubating the protein extract in 20 mM Tris-HCl (pH 7.4), 1 mM CaCl, 5 mM UDP-glucose and 20% (m/v) paramylon (Sigma) for 30 min at 25˚C followed by 90 min at 4˚C with gentle agitation. The proteins associated to the paramylon granule were centrifuged

at 15000 rpm at 4˚C for 15 min and the pellet was washed twice in buffer consisting of in 20 mM Tris-HCl (pH 7.4), 1 mM CaCl and 5 mM UDP-glucose. The paramylon granule with the entrapped proteins were incubated overnight at 4˚ C in 5X Laemmli loading buffer (5ml glycerol, 1g SDS, 2.56 ml β-mercaptoethanol, 2.13ml 0.5M Tris-HCl pH 6.8 and 5mg bromophenol blue). The denatured supernatant was removed for further purification by PAGE.

3.2.2.4 MOPS PAGE of the product entrapped protein

Partially purified proteins were separated at higher resolution using MOPS PAGE gels. Resolving gels were cast in 7 x 11 cm by 1.0 mm plates using a BioRad Protean minigel apparatus (BioRad Laboratories GmbH, Munich, Germany). Resolving gels (10%) consisted of 2.85ml 3.5X gel buffer (1.25 M bis-Tris-HCl (Bis(2-hydoxyethyl) aminotris (hydroxymethyl) methane, Sigma) pH 6.8), 3.33ml acrylamide:bis-acrylamide (30%:0.8%), 3.82ml H_2O, 100µl 10% APS (Ammonium persulfate) and 10µl TEMED. Stacking gels (4%) consisted 2.85ml 3.5X gel buffer, 1.7ml acrylamide:bis-acrylamide (30%:0.8%)(Sigma-Aldrich), 5.45ml H_2O, 100µl 10% APS and 10µl TEMED. A 5X high-MW running buffer (250mM MOPS, 250mM Tris, 5mM EDTA, 0.5% SDS and 5mM $NaHSO_3$ (sodium bisulfite)) was used for the electrophoresis. Electrophoresis was performed at room temperature at 150V.

Desalted partially purified protein samples were prepared in 5X Laemmli loading buffer (5ml glycerol, 1g SDS, 2.56 ml β-mercaptoethanol, 2.13ml 0.5M Tris-HCl pH 6.8 and 5mg bromophenol blue). Product entrapment samples were suspended and diluted in Laemmli loading buffer. Samples were incubated in loading buffer at 95˚C for 5 min. A pre stained PAGE Marker™ Prestained Protein Ladder (Fermentas #SM0671) was used as the molecular marker.

3.2.2.5 Staining of PAGE gels

Gels were fixed in a buffer (25% isopropanol (v/v) and 10% acetic acid (v/v)) for one hour and then rinsed in distilled water. The Colloidal Coomassie Blue Staining Kit (Invitrogen) was used to stain for one hour, and then destained with several washes of distilled water on a rotational shaker.

3.2.2.6 Western blots

Desalted partially purified protein and protein purified by product entrapment was blotted onto Nitrocellulose and Immobulin - PVDF from SDS PAGE and MOPS PAGE gels according to Sambrooke *et al*. (1989). Protein samples were prepared and denatured as described for PAGE gels and a PAGE Ruler™ Prestained Protein Ladder (Fermentas #SM 0671) was used as the molecular marker. Following electrophoresis, PAGE gels were pre incubated in transfer buffer (48mM Tris-HCl, 39mM glycine, 20% methanol and 0.0375% SDS). Separated proteins were transferred from PAGE gels onto membranes with the use of a Trans-Blot SD semi-dry electrophoretic transfer cell (BioRad Laboratories GmbH, Munich, Germany). Membranes were washed in TBST (20mM Tris-HCl pH 7.5, 150mM NaCl and 0.05% Tween 20) and blocked in blocking solution consisting of TBST and 1% BSA (Bovine Albumen (Fraction V), Roche) for two hours. An anti paramylon rabbit raised polyclonal primary antibody (University of Stellenbosch, South Africa), raised against proteins associated to the paramylon granule, was used as the primary antibody. The membranes were washed five times in TBST and incubated in 1% BSA and TBST solution with the secondary antibody in a 1:10000 dilution for one hour. The membranes were subsequently washed 5 times in TBST and rinsed with distilled water and incubated in a solution consisting of one NBT/BCIP Ready-to-use tablet (Roche) in 10ml distilled water, until colour development was observed. The membranes were washed in water to stop the colour development reaction.

3.2.3 Tryptic digestion and LCMSMS of partially purified paramylon synthase

3.2.3.1 Trypsin digestion of excised gel fragments

Following SDS-PAGE gel electrophoresis, protein bands stained with Page Blue™ (Fermentas) were excised from the gel according to Shevchenko *et al*., 1996. Gel fragments were excised and cut up into smaller fragments and dehydrated at room temperature for 10 min in acetonitrile. The fragments were dried in a speed-vac for 10 min and soaked in 150µl of 10mM DTT and 100mM NH_4HCO_3 for 1 h at 56˚C. Gel fragments were subsequently soaked in 150µl of 100mM NH_4HCO_3 and 55mM iodoacetamide for 45min in the dark at room temperature and then washed in 150µl of 100mM NH_4HCO_3 for 10 min at RT. The gel fragments were dehydrated in acetonitrile again for 10 min at RT and washed in 100mM NH_4HCO_3 before being dried for 10min in a speed-vac.

Gel fragments were incubated on ice in 35µl digestion buffer (12.5ng/µl trypsin (Promega sequence-grade modified porcine trypsin, #V511A) and 50mM NH_4HCO_3). The digestion buffer was removed

and the fragments were hydrated in 10µl of 50mM NH_4HCO_3 and cleaved overnight at 37˚C. Fragments were centrifuged for 1 min at 13,000 rpm and the supernatant was removed and 20µl of 20mM NH_4HCO_3 was added to the gel fragments and incubated for 10min at room temperature. The removed supernatant was added to the digested gel fragments with 25µl of 5% formic acid and 50% acetonitrile solution and incubated for 10 min at room temperature. The 5% formic acid and 50% acetonitrile was removed by centrifuging the tube for 1 min at 10,000g and the supernatant was saved and dried using a speed-vac.

3.2.3.2 LCMSMS

The digested peptides were sequenced at the Central Analytical Facility (University of Stellenbosch, South Africa). A CapLC coupled to a Waters API Q-TOF Ultima (Massachusetts, USA) was used to sequence the digested peptides which were seperated on a capillary LC coupled to the nano-spray (ESI) source of the tandem mass spectrophotometer (QTOF). The probable sequence identity of the peptides was determined using sequence information of collisionally induced dissociation of selected peptide ions against the database.

3.3 Transcriptome sequencing

The *E. gracilis* transcriptome was sequenced using 4µg highly purified cDNA (Section 3.1.1.1 – 3.1.1.4) with the genome sequencer FLX system (Roche 454, USA) at inqaba biotech, (Pretoria, South Africa). The *E. gracilis* genome data was obtained from the MC Field laboratory sequence database (http://web.me.com/mfield/Euglena_gracilis).

Results and Discussion

4.1.1 Construction, amplification, infection and expression of the cDNA library

Euglena gracilis liquid cultures were grown and harvested during exponential growth phase and paramylon synthesis after supplementation of fresh media containing glucose. Total RNA was extracted from harvested cells, quantified and tested for quality as described. The extracted RNA was further purified to obtain high quality mRNA that would express gene products and exclude rRNA. The mRNA was reverse transcribed into cDNA and separated by agarose gel electrophoresis. A gel

fragment of the resulting cDNA smear was excised that included cDNA fragments larger than 1000bp. These cDNA fragments were ligated into the pBluescript based library and packaged into empty phage particles. Phage particles were incubated with XL1-Blue MRF´ *E. coli* cells to form a primary library. The primary library was then amplified to make an amplified library by incubating the primary library with XL1-Blue MRF´ cells and plating them out, incubating and lysing them from which phages were washed and collected. The amplified cDNA phage library was then used for further screening. A mass excision library was constructed to generate a pBluescript based plasmid library. The library was grown from phage infected SOLAR cells an approximately 1ml at 1µg/µl of the mass excision library was purified.

4.1.2 Screening of the library

4.1.2.1 Screening of the phage library using an antibody

Nitrocellulose membranes were lifted from phage infected bacterial plates where proteins expressed by the phage library were bound. These membranes were screened using a polyclonal antibody raised against the proteins associated to paramylon granular membrane as the primary antibody. The membranes incubated on the plaques were marked for orientation and then blocked. The primary antibody was incubated in blocking solution as described and incubated with a secondary antibody conjugated with an alkaline phosphatase. The membranes were screened for alkaline phosphatase activity to determine the position of plaques expressing proteins that bound to the nitrocellulose membranes. In total, an estimated 186 000 plaques were screened on 116 membranes. Plaques that generated signals and that gave significantly darker signals than neighbour plaques after immunoblotting were identified and isolated through single clone excision, and used to directly infect secondary screens. However, secondary screens never produced a concomitant increase in the number of darker signals. Plaques that did not generate a darker signal were used as negative controls for secondary screens. Variations of exposure with the primary antibody or NBT/BCIP did not produce different results.

The primary antibody, to an extent non-specifically recognized all plaques that formed. It was assumed that despite this, paramylon granule related proteins expressed would have produced darker signals than the non specific plaques. As the polyclonal primary antibody was raised in rabbits, anti *E. coli* antibodies would also be present in the primary antibody serum. During cell lysis, bacterial proteins would also have been bound to the membranes producing a background signal. If

paramylon synthase related proteins were not expressed significantly to generate a darker signal, any positive signals would have been marginalized.

As a significant number of plaques were screened, it can be speculated that problems would have been caused by the non specific recognition of the antibody or possibly due to the expression of the membrane proteins which have different folding conformations and epitopes within the bacterial system. Later experiments confirmed the polyclonal antibody's specificity for solubilized paramylon synthase proteins after isolation from the membranes.

4.1.2.2 Screening of the library using radio-labeled substrate

Nitrocellulose membranes were lifted from phage infected plates where proteins expressed by the phage library were bound. These membranes were screened using the radio-labeled [^{14}C] UDP-glucose for substrate (Bäumer *et al*., 2001; van der Merwe, 2007). Approximately 33,000 plaques were screened in this way. Protein products expressed during phage lysis were blotted onto the membranes. The blocked membranes were incubated with limited [^{14}C] UDP-glucose and the membranes were stored with phosphor imager screens for up to seven days. Different blocking solutions as well as exposure times were used but did not have significant differences. Images were obtained from the membranes and darker signals, potentially positive clones caused by [^{14}C] UDP-glucose accumulation, which appeared on the membranes were used to identify the corresponding plaques on the plate. These phages were excised, stored and used to directly inoculate secondary screening experiments. Plaques that did not give signal were used with secondary screens as a control. However, secondary screens often did not yield a greater number of positive clones the primary screens and membranes with negative controls often yielded a comparative amount of false positive signals. During secondary screens, membranes that appeared to have a considerably greater number of positive clones relative to the other membranes were then compared to the plaques on the plates and the phages were isolated through single clone excision. During one secondary screen, one membrane showed a considerably greater number of signals compared to the other membranes in the secondary screen and the negative control. Corresponding plaques were purified through single clone excision and the pBluescript vectors that were purified were sequenced. If the signals were caused by a peptide due to affinity for [^{14}C] UDP-glucose it would be expected that the clones excised during the secondary screen would all have similar sequences. However, the sequence data was analysed using NCBI BLAST where none of the excised clones shared sequence similarity to glycosyl transferases or were similar to each other (Table 6). The presence of specific *E. gracilis*

genes, such as tubulin, elongation factors and cytoskelatal proteins, indicate that the library was at least amplified but that the phages were expressing non specific gene products.

Table 6: BLASTx results of false positive clones of the [^{14}C] UDP-glucose radio-labeled phage screens

Clone nr.	Description of BLASTx hit result	E-value	NCBI Accession nr.
1	60S ribosomal protein L2 [Trypanosoma cruzi]	2.0E-71	ref|XP_816366.1|
2	cytoskeletal protein [Euglena gracilis]	3.0E-13	gb|AAB23241.1|
8	receptor of activated kinase C 1 [Chlamydomonas reinhardtii]	2.0E-14	ref|XP_001698065.1|
9	beta-tubulin [Euglena gracilis]	2.0E-65	gb|AAK37834.1|AF182558_1
10	elongation factor 1 alpha [Euglena gracilis]	5.0E-43	gb|ACO50110.1|

Due to the amount of false positive signals yielded as well as the difference in sequence data from clones that gave signals in secondary screens, it seems that the signals observed were not caused by peptides with affinity for [^{14}C] UDP-glucose but rather due to non-specific binding or possibly the formation of [^{14}C] UDP-glucose artifacts on the membranes. Incubation with insufficient amount of [^{14}C] UDP-glucose to provide a signal, or due to too low expression of the protein may also explain the lack of positive signals. Changes in enzyme conformation due to expression in the bacterial system may also influence the substrate binding affinity of the membrane-bound paramylon synthase enzymes.

4.1.2.3 Functional screening of the mass excision library using aniline blue

Functional screening was performed based on the affinity of analine blue for β-1,3-glucan polymers. Analine blue stains β-1,3-glucans yellow under ultraviolet light where colonies transformed with the mass excision library, containing cDNA inserts that express β-1,3-glycosly transferases, would be expected to stain positively for the product. However, problems with this system is that it relies on expression of active β-1,3-glycosyl transferase, that regulatory peptides are not essential for minimal protein expression, or that the membrane bound paramylon synthase subunits would be functionally expressed in the bacterial cytosol, or that the stain would be able enter the bacterial cell. Some of these problems would be overcome by lysing the cells with lysozyme but this would also create problems during the wash steps. No colonies stained yellow under ultraviolet light and it was assumed that if any paramylon synthase genes were expressed they were not functional in the bacterial system.

4.1.2.4 Colony library screens

Similarly to the functional screen, the mass excision library containing cDNA inserts from *E. gracilis* was transformed into the SOLAR *E. coli* strain. As the library was pBluescript based, the transformed bacterial colonies would express any inserted cDNA. Colonies were blotted onto nitrocellulose membranes, replicated, fixed and then blocked with a blocking solution that included lysozyme. The membranes were not washed after lysis although fresh blocking buffer was added during incubation with the primary antibody. It was thought that the remaining lysate on the membranes and in the Petri dishes would be enough to reduce non specific binding of *E. coli* proteins. However, non specific signals still appeared in the screens. Secondary screens were conducted, using colonies from replica membranes, which gave considerably darker signals. Colonies that did not give darker signals were used as negative controls. None of the colonies during the second rounds of screening gave significantly darker signals relative to the control.

4.1.3 Screening of cDNA using PCR

Isolated cDNA from *E. gracilis* was used as template for PCR reactions using degenerate and non degenerate primers designed from a partially purified peptide, with UDP-glucose binding affinity, through SDS-PAGE and LCMS, from protein partially purified and solubilized from the paramylon granular membrane (van der Merwe, 2007). Primers were redesigned based on that peptide sequence but with a 4 fold degree of degeneracy for this study. The peptide sequence from a 37 kDa peptide, which had UDP-glucose binding affinity, showed identity to the fungal glucan synthases of *Debaryomyces hanseii* and KRE6 β-1,6-glucan synthase of *Candida albicans* and *Saccharomyces cerevisiae.* The newly designed primer sequences included conserved WN amino acids on the N-terminal which were derived from the *Debaryomyces hanseii*, which increased the length of the primer without increasing the degeneracy. The newly designed degenerate and non-degenerate primers were used to screen purified cDNA using PCR. Conserved domains derived from β-1,3-glucan synthases of several organisms were also used to design degenerate primers (Table 4). These primers were used with oligo(dT) primers to screen *E. gracilis* cDNA. Several bands were amplified and excised and purified from agarose gels, cloned and sequenced. When the sequences were screened using NCBI BLASTx, no glycosyl transferase related sequences were identified.

The mass excision library was screened using the conserved domains primers (CD) (Table 3). As the mass excision library was designed in pBluescript, the inserted cDNA is flanked by both T3 and T7 primers. Due to the directional insertion into the vector (Zap cDNA Synthesis kit protocol), the T3

primer would be able to be paired with a gene specific reverse primer. The conserved, degenerate and non-degenerate reverse primers were paired with the T3 primer and the forward primers were paired with the T7 primer which were used to amplify DNA from the mass excision library. Several bands per PCR, due to the nature of the cDNA library, were amplified, cloned, purified and sequenced. Sequence data (Table 7) from the amplified bands of the mass excision library did not show identity to known glycosyl transferases.

Table 7: PCR results of cDNA and the mass excision library amplified and sequenced.

Primer pair	BLASTx description of the sequence	Accession number	E-value
cDNA template			
rev CD1 - oligo(dT)	aldehyde dehydrogenase (acceptor) [*Trichodesmium erythraeum* IMS101]	ref|YP_722268.1|	9.0E-43
rev CD2 - oligo(dT)	hypothetical protein LOC100187597 [*Nasonia vitripennis*]	ref|NP_001127800.1|	3.1E+00
rev CD3 - oligo(dT)	Receptor of activated protein kinase C 1A, [*Ostreococcus lucimarinus*]	ref|XP_001421096.1|	6.0E-20
rev CD4 - oligo(dT)	PREDICTED: similar to ribosomal protein L8 [*Strongylocentrotus purpuratus*]	ref|XP_796001.1|	3.0E-55
rev CD5 - oligo(dT)	beta-tubulin [*Euglena gracilis*]	gb|AAK37834.1|AF182558_1	4.0E-73
fwd non deg - oligo (dT)	ubiquitin-protein ligase, putative [*Trypanosoma brucei gambiense*	emb|CBH13171.1|	1.8E+00
fwd non deg - oligo (dT)	putative ribosomal protein L15 [*Diaphorina citri*]	gb|ABG81972.1|	1.0E-35
rev deg - oligo (dT)	Adenosylcobalamin-dependent ribonucleoside-triphosphate reductase [*Euglena gracilis*]	sp|Q2PDF6.1|RTPR_EUGGR	6.0E-45
rev deg - oligo (dT)	PREDICTED: thrombospondin, type I, domain containing 7B isoform 2 [*Oryctolagus cuniculus*]	ref|XP_002712172.1|	1.3E+00
fwd deg - oligo (dT)	ADP-ribosylation factor 1 [*Trypanosoma congolense*]	gb|ABT17155.1|	4.0E-44
Mass excision library template			
rev CD1 - T3	cytoskeletal protein [*Euglena gracilis*]	emb|CAA78364.1|	9.0E-13
rev CD2 - T3	ATP-dependent Clp protease ATPase subunit [*Geobacillus kaustophilus* HTA426]	ref|YP_145931.1|	5.5E+00
rev CD3 - T3	similar to activated kinase C receptor [*Trypanosoma carassii*]	gb|ABS01292.1|	5.0E-17
rev CD4 - T3	5-methyltetrahydropteroyltriglutamate/homocysteine S-methyltransferase [*R. pickettii*]	ref|YP_001893059.1|	2.0E-22
rev CD5 - T3	beta-tubulin [*Euglena gracilis*]	gb|AAK37834.1|AF182558_1	3.0E-72
fwd CD6 - rev CD7	elongation factor 1 alpha [*Euglena gracilis*]	gb|ACO50110.1|	4.0E-74
fwd CD7 - rev CD2	60S ribosomal protein L12 [*Sclerotinia sclerotiorum* 1980]	ref|XP_001586492.1|	2.0E-08
fwd CD2 - rev CD1	5-carboxymethyl-2-hydroxymuconate Delta-isomerase [*Geobacter* sp. M21]	ref|YP_003020495.1|	2.0E-12
fwd CD1 - rev CD3	predicted protein [*Thalassiosira pseudonana* CCMP1335]	ref|XP_002294414.1|	7.0E+00
fwd CD4 - rev CD5	predicted protein [*Physcomitrella* patens subsp. patens]	ref|XP_001772298.1|	1.0E-05
T3 - rev CD1	spore coat protein (outer) [*Bacillus subtilis* subsp. subtilis	ref|NP_391486.1|	8.3E-02
T3 - rev CD2	heat shock protein 60 [*Euglena gracilis*]	emb|CAA65238.1|	8.0E-41
T3 - rev CD3	plasma memebrane H+-ATPase [*Plantago major*]	emb|CAL35828.1|	5.0E-04
T3 - rev CD4	ribosomal protein L13A [*Schizophyllum commune* H4-8]	ref|XP_003038672.1|	3.0E-14
T3 - rev CD6	ribulose-bisphosphate carboxylase [*Euglena gracilis*]	emb|CAA55779.1|	5.0E-99
T3 - rev CD7	6-phosphogluconolactonase [*Chryseobacterium gleum* ATCC 35910]	ref|ZP_07087499.1|	6.9E+00
T7 - fwd CD1	spore coat protein (outer) [*Bacillus subtilis* subsp. subtilis	ref|NP_391486.1|	1.4E-01
T7 - fwd CD2	eukaryotic initiation factor 4E, putative [*Phytophthora infestans* T30-4]	ref|XP_002907026.1|	2.0E-23
T7 - fwd CD3	vacuolar ATP synthase [*Leishmania infantum* JPCM5]	ref|XP_001465476.1|	2.0E-31
T7 - fwd CD4	succinyl-CoA synthetase subunit beta [*Rickettsia typhi* str. Wilmington]	ref|YP_067379.1	4.0E-04
T7 - fwd CD7	hypothetical protein [*Trichomonas vaginalis* G3]	ref|XP_001322505.1|	4.0E-04

4.2 Protein purification

4.2.1 Paramylon synthase crude protein extraction

Euglena gracilis cells, grown under heterotrophic conditions, were centrifuged and disrupted by sonication on ice in buffer containing protease inhibitors and DTT. After disruption the paramylon granules were separated and washed of the cellular membranes and cell debris by centrifugation. After the paramylon granules were washed, the paramylon synthases and membrane associated proteins were solubilized from the granule membranes with the detergent CHAPS. Activity was determined by Schiff staining native PAGE. Previous studies have shown paramylon synthase activities from crude protein extracts using UDP-glucose assays, Schiff staining and Coomassie colloidal blue staining (Bäumer *et al*., 2001; van der Merwe, 2007).

4.2.2 FPLC, PAGE separation, product entrapment and immunoblotting of partially purified paramylon synthase proteins

4.2.2.1 FPLC of paramylon synthase proteins

The polyclonal antibody was purified using an ACTA prime FPLC and a HiTRAP Protein A column. Elution of the polyclonal antibody from the column successfully separated the polyclonal antibody from serum contaminants and proteins (Figure 4). The purified polyclonal antibody was coupled to a CNBr-activated sepharose column and partially purified protein extract was run through the column and allowed to bind to the antibody based on specific affinity for the polyclonal antibody. The resultant peaks indicated that protein that had affinity for the coupled antibody was eluted when the binding buffer was replaced with the elution buffer (Figure 5). The first peak represented all other non-specific proteins without antibody affinity while the second minor peak represented the protein with antibody affinity. As the total protein loaded was ~5mg the relative peak sizes suggest a very low concentration of proteins with antibody binding specificity would have been eluted which was not detectable in subsequent ammonium sulfate precipitation purification steps.

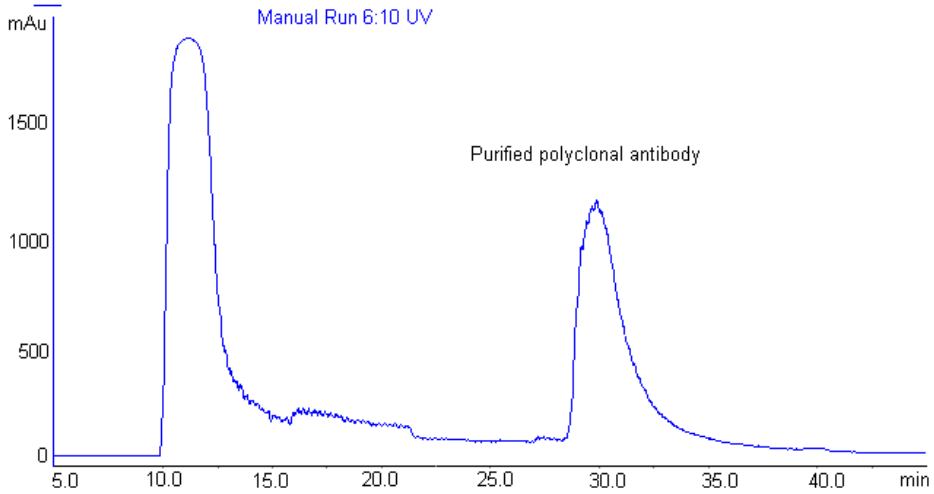

Figure 4: FPLC purification of polyclonal anti-paramylon synthase antibodies. The antibodies were eluted at 30 min before being coupled to CNBr-activated sepharose.

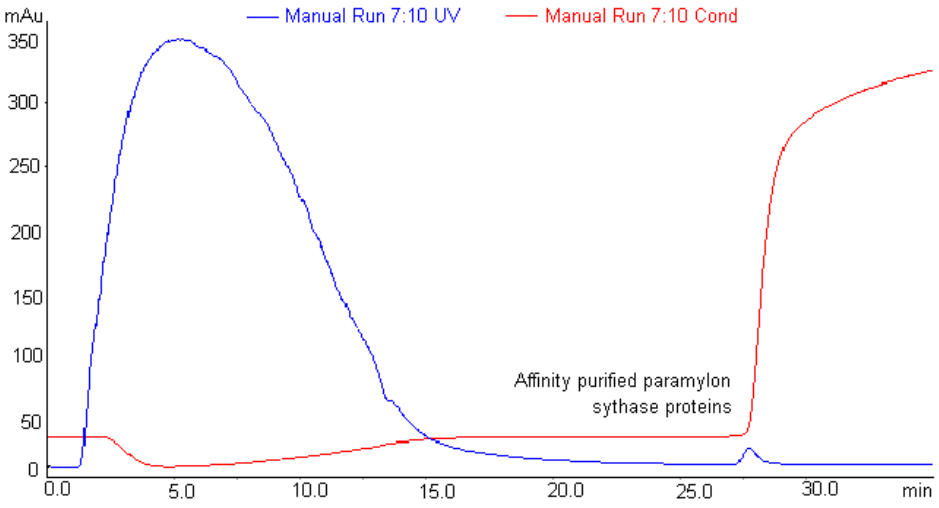

Figure 5: FPLC purified paramylon synthase proteins were purified through the polyclonal antibody bound CNBr-sepharose column after the binding buffer was exchanged with the elution buffer after non specific proteins were washed out.

4.2.2.2 Detection of in gel paramylon synthase activity

Paramylon synthase activity was detected in protein solubilized from the granule membranes by separating solubilized undenatured protein on native PAGE followed by staining the gels in Schiff stain solution. Solubilized glucan synthases are known to remain stable and active in gels (Thelen and

Delmer, 1986). Paramylon as a primer was not incubated in the reaction with the protein extract although Ca^{2+} cation cofactors and UDP-glucose as substrate were added during incubation.

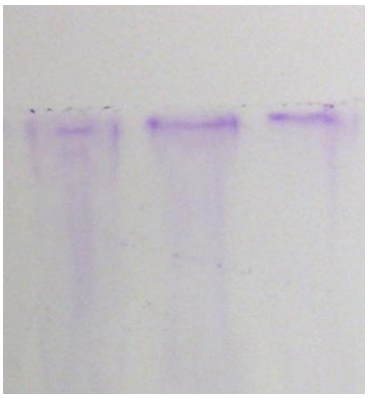

Figure 6: Schiff stained Native PAGE of in-gel paramylon synthase activity. Partially purified CHAPS solubilized protein was loaded undenatured into the three lanes. Schiff staining of the gel showed a large presumably ~670 kDa protein complex (Bäumer et al, 2001). Control gel did not stain a product (Data not shown).

Schiff staining of the native PAGE showed product formation by a high molecular weight protein complex migrating only slightly into the resolving gel (Figure 6) but not in the control (Data not shown). As this corresponds with previous results (van der Merwe, 2007) and as *E. gracilis* does not produce other carbohydrate polymer products such as cellulose, glycogen or starch the product was taken to be paramylon. The paramylon synthase complex was previously determined to be 670 kDa using a similar method (Bäumer *et al*., 2001).

4.2.2.3 Paramylon synthase partial purification through product entrapment

CHAPS solubilized crude protein extract was dialyzed to further purify and concentrate the protein extract. The partially purified protein was quantified. A method of protein purification, specifically for β-glucan synthases has been through product entrapment (Kang *et al*., 1984) where many of the β-1,3-glucan synthases from plants and some fungi have been successfully partially purified and enriched using this method. Product entrapment of protein relies on the association of the enzyme complex with the product. Partially purified protein is incubated with the purified product and allowed to associate with the insoluble product. Insoluble purified paramylon (SIGMA) was used as the product which was incubated at room temperature with dialyzed protein extract, Ca^{2+} and UDP-glucose substrate. In literature, several unidentified proteins were non-specifically purified with the

β-1,3-glucan synthase proteins. As several functionally different proteins are involved in the synthesis of paramylon it was expected that several proteins would be enriched with the β-1,3-glucan synthase proteins along with non-specifically enriched proteins. Following incubation, the insoluble protein-paramylon entrapment was separated from the unbound proteins using a filtered spin column. The filter of the spin column with the residual protein-paramylon entrapment was removed and separated using MOPS-PAGE (Figure 7, p 40).

4.2.2.4 MOPS-PAGE of the product entrapped protein

The protein-paramylon entrapment was separated using MOPS-PAGE. The protein-paramylon entrapment was denatured by heat and incubation with SDS loading buffer. The SDS loading buffer diluted the purified protein and the denatured protein was spun down to remove the insoluble paramylon. The MOPS-PAGE separated several protein bands with high resolution, of which several appeared enriched after staining with Coomassie. Although many faint bands were also separated. Previous literature identified the sizes of the β-1,3-glucan synthase catalytic subunits using radio-labeled substrates or immune-precipitation. Many of the faint bands were then excluded as non-specific proteins.

Previous literature (Bäumer *et al*., 2001) identified two bands, 55 and 37 kDa which bound ^{32}[P] UDP-glucose, purified with sucrose density gradient ultracentrifugation. Coomassie staining of the same protein factions showed several unidentified bands separating at approximately 20, 60, 62 and 72 kDa as well as the 55 and 37 kDa bands. These other bands were never investigated, as the proteins did not bind UDP-glucose, although may still be involved in the glucan synthase complex or associated with the membrane as structural or transport proteins. In more recent studies in this lab (van der Merwe, 2007), similar experiments to Bäumer's group identified proteins separating in a similar pattern for Coomassie stained PAGE gels as well as for ^{32}[P] UDP-glucose in-gel assays, except that a protein of approximately 80 kDa also bound ^{32}[P] UDP-glucose but was never further analyzed. Although these experiments separated and purified the proteins using different methods, similar patterns were observed with those purified and enriched using product entrapment.

4.2.2.5 Western Blot and gel excision of paramylon synthase proteins

Product entrapped peptides were seperated using MOPS-PAGE where separate gels were stained with Coomassie colloidal blue and immunoblotted using a primary antibody raised against

membrane proteins associated to the granule (Figure 7). The western blot identified several proteins seperated by product entrapment purification which correlated to proteins enriched on the MOPS-PAGE gel. The proteins that were correlated with the bands on the western blot were excised from the MOPS-PAGE gel and treated with proteases. Seven bands were excised from the MOPS-PAGE gel of approximately 37, 55, 60, 62, 72, 80 and 95 kDa.

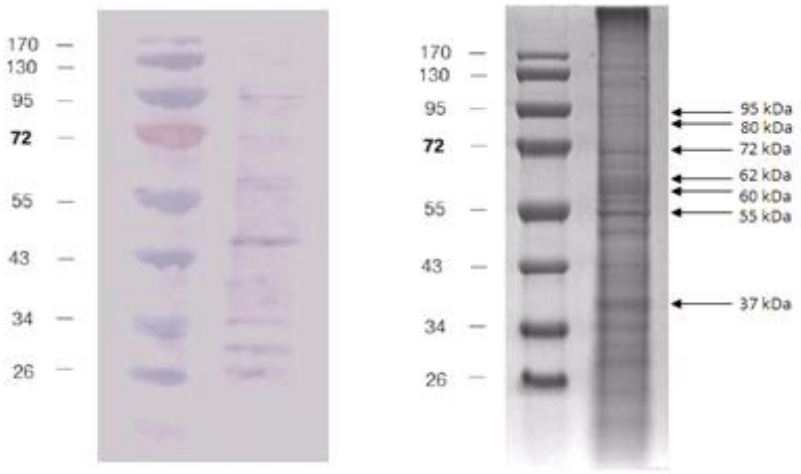

Figure 7: Western Blot (left) analysis of partially purified product entrapt proteins associated to the paramylon granule (right lanes). Seven proteins were separated with MOPS-PAGE (right), which corresponded to the western blot, were excised from the gel and digested using trypsin.

4.2.3 LCMSMS

The excised proteins were digested and short peptide sequences were predicted from each of these proteins. The results of the digested peptides were analyzed using the MASCOT program where each of the excised proteins from the partially purified from the paramylon granular membrane was digested, separated and grouped into queries. Each query contained predicted short peptide sequences which were analysed using BLAST and searched against the protein database (BLASTp) of NCBI where the NCBI reference numbers and the specific gene names were recorded in addendum A. For each short peptide, the first 100 BLAST hits were searched and the relevant genes were recorded, specifically β-1,3-glucosyl transferases, glycosyl transferase gene families and calcium and UDP-glucose binding proteins. Of approximately 900 predicted peptide queries, 289 peptides showed identity or partial identity to known glycosyl transferases. As β-1,3-glucan synthases fall under glycosyl transferase family 2 (GT2), with mannosyl transferases, chitin synthases and cellulose synthases and all having common GT-A and GT-B folding domains, predicted query peptides with

similar identities to GT-2 glycosyl transferases were selected. NCBI BLAST results did not usually have complete identity with the query peptides and the query peptides showed similar identity to many non-specific proteins with similar or even higher degrees of identity. E-values were recorded but were relatively very high due to the nature of the short peptide sequences.

4.3 Sequence analysis of the *E. gracils* transcriptome and genome

4.3.1 Sequencing of the *Euglena gracilis* transcriptome

The *E. gracilis* transcriptome was partially sequenced using the Genome Sequencer FLX system. High quality RNA was extracted from *E. gracilis* liquid cultures during exponential growth and paramylon synthesis. The total RNA was extracted and quantified from which mRNA was further purified by selecting mRNA sequences with poly-(T) tails most likely to express gene products. The purified mRNA was quantified and the quality of the sample was controlled using agarose gel electrophoresis. The mRNA was converted into double stranded cDNA and quantified and qualified (Figure 8). The prescribed amount of cDNA was purified and sent for sequencing.

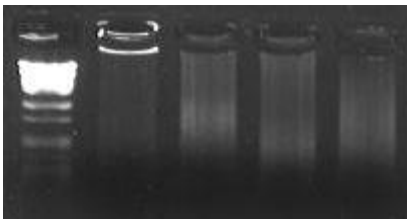

Figure 8: Purified double stranded cDNA smears (lanes 2-5) were pooled and sequenced using the genome sequencer FLX system.

The sequenced cDNA was assembled and arranged into overlapping reads called contigs using CLC genomics workbench. Normally, query transcriptomes are characterized against well characterized reference transcriptomes by directly comparing the generated FASTA sequences. Unfortunately in the case of *Euglena*, no reference sequence libraries are available and only limited sequence information is available.

The approach in this study was to directly sequence the transcriptome and BLAST annotate the available contigs to identify any glycosyl transferases, specifically UDP-glycosyl transferases, β-1,3-glycosyl transferases as well as GT-2 glycosyl transferases. Paramylon synthase genes most likely

originated from genes inherited through the secondary endosymbiotic event (Martin *et al*., 1993; Archibald *et al*., 2003; Timmis *et al*., 2004) as β-1,3-glucans do not appear in any kinetoplastid while they do appear in the forms of callose in plants (Samuels *et al*., 1995) and as cell wall constituents in fungal systems (Cabib *et al*., 1987). It is highly likely that paramylon synthase genes would have evolved from cellulose, callose and chitin synthases as opposed to the prokaryote β-1,3-glucans or kinetoplastid glycoproteins.

ORF's for potential prokaryotic and eukaryotic genes were identified from the contigs using the BLASTx function on CLC Genomics Workbench (contig assembly is summarized on Table 8 and Figures 9 and 10), including a pre-existing *Euglena* EST database (tbestdb, http://tbestdb.bcm.umontreal.ca), and arranged into contig queries with the reference NCBI accession numbers, number of reads, greatest read length and the lowest E value (data not shown). None of the contigs identified through BLASTx showed any identity to glycosyl transferases or for Ca^{2+} and UDP-glucose binding genes. With such a low number of contigs (1179), which only covered a fraction of the entire transcriptome, no glycosyl transferases were identified although many of the genes identified coded for hypothetical proteins and proteins of unknown function as was expected.

Following from that approach, the short peptide sequences (appendix A) obtained from partially purified proteins associated to the paramylon granule as well as the conserved domains from β-1,3-glucan synthases were used to screen against the partial transcriptome directly. The contigs were converted into ORF's in FASTA format using the GeneMark heuristic model (Besemer and Borodovsky, 1999). Both eukaryote and prokaryote ORF's were converted into protein FASTA sequences and used as a database to screen against using the paramylon synthase short peptide fragments (appendix A). As the peptides were expected to have complete identity with the paramylon synthase proteins, they were used directly to screen the ORF's. However, none of the peptide sequences matched any of the available ORF's.

Table 8: Contig assembly of the transcriptome

Assembled reads		**Contig count**	
Read count	2960	Contig count	1179
Mean read length	746.52	Mean contig length	702.15
Total read length	2209691	Mean coverage	2

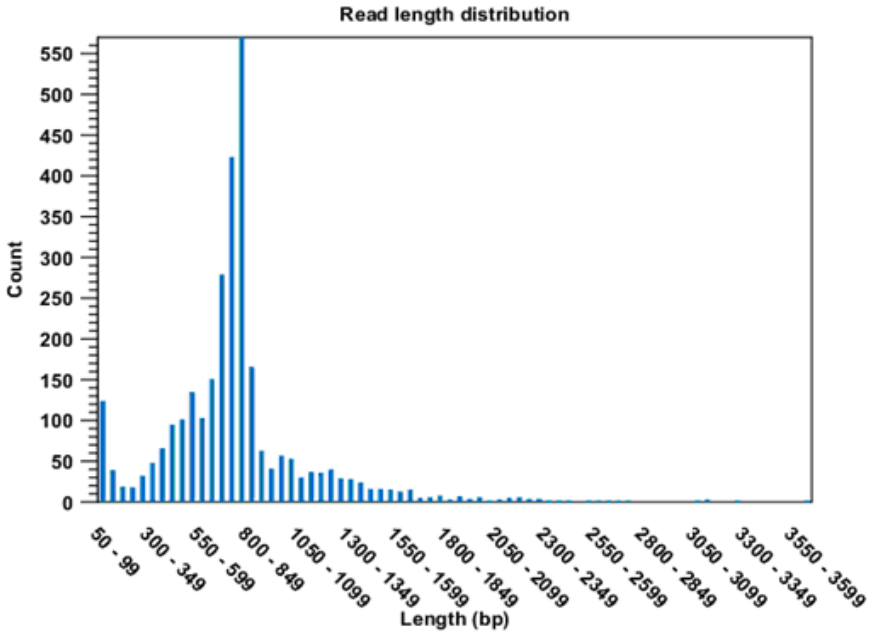

Figure 9: Read length distribution of the sequenced transcriptome.

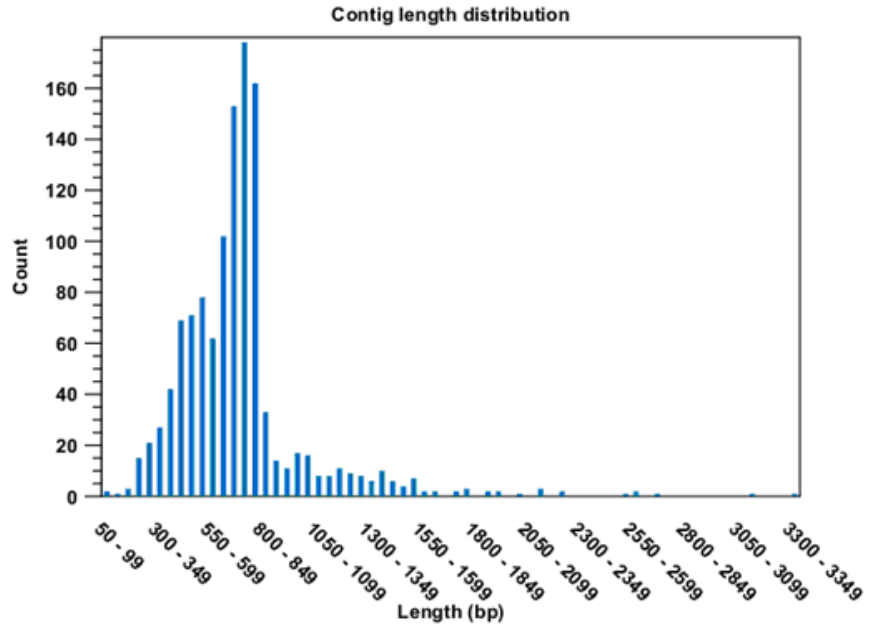

Figure 10: Contig length distribution of the sequenced transcriptome.

The absence of any glycosyl transferases from the transcriptome sequences (data not shown), with the exception of an α-1,6-mannosyl transferase from a different GT-family, where glycosyl transferases are estimated to account for about 1% of universal ORF's (Coutinho *et al.*, 2003) gives an indication of the low read yield. Although a great number of predicted short peptide fragments were identified with identity to glycosyl transferases, they all would be derived from the same proteins.

4.3.2 BLASTing the partially completed *Euglena gracilis* genome sequence

During the course of this study, a *E. gracilis* genome sequencing project was completed and released. The genome sequencing project was aimed at phylogenetic sequencing studies and was released on their website (http://web.me.com/mfield/Euglena_gracilis). The assembled contigs were obtained and BLAST searched using BLASTx and recorded. Initially, the short predicted peptide sequences were used to screen the transcriptome similar to the sequenced transcriptome of this study. Peptide sequences that showed some similarity to the contigs were recorded (Table 9 and 10), particularly where two peptides matched the same contig in the same reading frame. These contigs were BLASTed but did not show identity to any glycosyl transferase sequences. Although two partial sequences NFVNGL and LPPPPRTEL of the query peptide sequences, where the adjacent numbers designate nucleotide position on the contig subject sequences, followed on from each other. As the query LCMSMS peptides are only predicted sequences that were identified as possible glycosyl transferases using BLASTp, there is room for error occurring both with peptide prediction as well as BLAST identification. Many of the query peptides showed some identity with the contigs however most of the contig sequences have relatively short sequence lengths possibly resulting in predicted peptide pairs to be missed. Due to the lack of reference proteins, it may also be possible that the peptide sequences covered variable regions among β-glycosyl transferases and were not identified by BLAST.

Glycosyl transferases are highly variable between organisms and only share or partially share specific motifs and domains as described previously. Without the specific domains being present in the contig queries it is difficult to determine whether the contig is or is not a β-1,3-glycosyl transferase. As the highly conserved GT-2 motifs consist of only three or four amino acids and sometimes as single amino acids, it becomes very difficult to predict, confirm or rule out the contig query sequences that show identity to variable regions of the subject GT-2 glycosyl transferase proteins without further sequencing, cloning and screening for activity. Due to the nature of the assembled transcriptome, many of the contigs are incorrectly assembled having no consensus sequences, repeated and have sequence substitution and frame shift errors. When previously identified *E. gracilis* genes on NCBI were BLAST searched against the *E. gracilis* transcriptome, the genes would be separated over several contigs with minor substitution and deletion sequence errors. Sequence errors causing frame shifts would cause potential positive sequences to be missed using BLASTx.

The sequenced genome consisted of 29613 assembled contigs assembled from ~4 million reads which were BLAST searched using the NCBI BLASTx function of CLC Genomics Workbench. All BLAST results were filtered for “glycosyl transferases”, “glucan”, “cellulose” and “chitin” synthases and recorded (Data not shown). GT-2 sequences with the lowest E-values and highest identities were recorded along with the conserved domains of the subject sequence (Table. 10).

Several potential β-1,3-glucan synthase partial sequences were identified along with GT-2 glycosyl transferase and cellulose synthase sequences using BLASTx. Candidate sequences which partially aligned with GT-2 glycosyl transferases are represented with their corresponding E-values, related conserved domains and motifs (Table 10). Contigs that had significant identity to β-1, 3-glucan synthases and GT-2 glycosyl transferases were aligned (Table 11). Two contigs showed significant partial identity with β-glycosyl transferases; contig 11792 (Figure 13, p 49) and contig 11487 (Figures 14 and 15, p 51). The expect values of the alignments are relatively high and the queries align only partially with the subject sequences.

Table 9: Predicted peptide sequences aligned with the contigs of the sequenced genome

Peptide sequence	Protein	Peptide BLAST description	Contig	E-value	ORF	Contig sequence match
LSSSASPAPTK	80 kDa	ref|XP_001568074.1| GTP-binding elongation factor tu protein [*Leishmania braziliensis*]	contig0243	9.8	+1	130- SSSASPA -150
GPSSPSSSADACR	35 kDa	ref|ZP_06276423.1| β-1,3-glucan synthesis-like protein [*Streptomyces sp*.]	contig0243	4.0	+1	157- PSSPASASGAC -189
MLIPVMRWQR	80 kDa	ref|YP_001634862.1| glycosyl transferase group 1 [*Chloroflexus aurantiacus* J-10-fl]	contig0650	7.3	+1	241- IPVLRW -258
EVRIGSASVR	35 kDa	ref|YP_003013567.1| Peptidoglycan glycosyltransferase [*Paenibacillus sp*. JDR-2]	contig0650	3.0	+1	520- VRIGSANV -543
KYYDDSPWQR	80 kDa	ref|ZP_01092700.1| putative glycosylhydrolase [*Blastopirellula marina* DSM 3645]	contig0102	5.5	-2	3037- DDSPWLR -3017
WDYAVVASGGFFIAGK	50 kDa	ref|YP_152483.2| peptidoglycan synthetase [*Salmonella enterica* subsp. ATCC 9150]	contig0102	9.4	-2	1930- AVVASGG -1910
ISSHSLEELAAELQISKEK	62 kDa	ref|YP_001450314.1| glycosyltransferase [*Streptococcus gordonii str. Challis* substr. CH1]	contig0179	5.1	-3	555- SHSLDPLSAE -526
FTRDWTK	60 kDa	ref|YP_001404698.1| glycosyl transferase, group 1 [*Candidatus Methanoregula boonei*]	contig0179	1.2	-3	462- TRDWTK -445
QVNSIYGKFSASWK	60 kDa	ref|YP_003182856.1| glycosyl transferase family 51 [*Eggerthella lenta* DSM 2243]	contig0075	7.1	+2	548- NSIFQKFSA -574
GTGCSPDLPPPSRAEMGR	50 kDa	ref|YP_001537113.1| glycosyl transferase group 1 [*Salinispora arenicola* CNS-205]	contig0075	6.9	+2	140- SPDLPP -157
GLGMLGWVFFPPREDLSGGIDR	35 kDa	ref|ZP_03997417.1| glycosyltransferase [*Halogeometricum borinquense* DSM 11551]	contig0470	3.7	+3	93- PPREDL -110
SGFFSHCCLSFPSK	50 kDa	ref|ZP_05724441.1| glycosyl transferase group 1 [*Dickeya dadantii* Ech586]	contig0470	4.0	+3	27- FGNCCFSFP -53
		ref|ZP_06115144.1| glycosyl transferase, group 1 [*Clostridium hathewayi* DSM 13479]	contig0210	9.6	-2	228- CCLFFPS -208
GPSSPSSSADACR	35 kDa	ref|ZP_06276423.1| β-1,3-glucan synthesis-like protein [*Streptomyces sp*. ACT-1]	contig0210	7.2	-2	543- SPSTSPAACR -514
VKGNFVNGLPDGTLEK	50 kDa	ref|YP_679052.1| glycosyltransferase-S [*Cytophaga hutchinsonii* ATCC]	contig2812	7.0	+2	68- NFVNGL -85
		ref|ZP_04061534.1| glycosyltransferase-I [*Streptococcus salivarius* SK126]	contig0852	7.0	-2	168- NFVNGL -151
			contig0684	7.0	+1	574- NFVNGL -591
GTGCSPDLPPPSRAEMGR	50 kDa	ref|YP_001537113.1| glycosyl transferase group 1 [*Salinispora arenicola* CNS-205]	contig2812	6.9	+2	86- LPPPPRTEL -112
			contig0852	6.9	-2	150- LPPPPRTEL -124
			contig0684	6.9	+1	592- LPPPPRTEL -618
			contig1856	6.9	-3	216- DLPPPS -199
GAFHTVSLQKYSK	35 kDa	ref|ZP_03395721.1| glycosyl transferase, group 2 family protein [*Pseudomonas syringae*]	contig1856	7.2	-3	21- LQKYSK -4

Table 10: BLASTx results of the genome contigs aligned with two predicted peptide sequences.

Contig	Contig BLASTx description	E-value
contig02437	no hits for specific reading frame	
contig06506	no hits for specific reading frame	
contig01027	no hits for specific reading frame	
contig01797	|XP_002999687.1| predicted protein [*Verticillium albo-atrum* VaMs.102]	5.7
contig00754	no hits for specific reading frame	
contig04704	|YP_890301.1| DNA repair protein RadA [*Mycobacterium smegmatis* str. MC2 155]	3.4
contig02102	no hits for specific reading frame	
contig28129	|XP_002564567.1| Pc22g05320 [*Penicillium chrysogenum Wisconsin* 54-1255]	3.4
contig08522	|XP_002564567.1| Pc22g05320 [*Penicillium chrysogenum Wisconsin* 54-1255]	3.4
contig06849	no hits for specific reading frame	
contig18568	no hits for specific reading frame	

Table 11: Contig sequences from the sequenced genome with partial identity to GT-2 glycosyl transferase protein sequences.

Contig	BLASTx description of similar proteins and their accession numbers	E-value	Domain	Motif	Domain descriptions of the subject sequence
Contig 2502	|YP_002975125.1| cellulose synthase subunit B [*Rhizobium leguminosarum*]	3.00E+00	pfam03170		BcsB; Bacterial cellulose synthase subunit
			PRK11114		cellulose synthase regulator protein
Contig 2607	|YP_480685.1| glycosyl transferase family protein [*Frankia sp*. CcI3]	3.00E+00	cl11394		Glyco_tranf_GTA_type; Glycosyltransferase family A (GT-A)
Contig 3502	|XP_002793109.1| 1,3-beta-glucanosyltransferase gel2 [*Paracoccidioides brasiliensis* Pb01]	9.20E+00	cl12144		glycosyl hydrolase family 5
Contig 5589	|CBJ30317.1| Cellulose synthase (UDP-forming), family GT2 [*Ectocarpus siliculosus*]	4.00E+00	cd06421		CESA_CelA like cellulose synthase GT-2
Contig 9141	|YP_001682980.1| glycosyl transferase family protein [*Caulobacter* sp. K31]	7.00E-02	cd00761	DXD	Glyco_tranf_GTA_type; Glycosyltransferase family A (GT-A)
Contig 10917	|ZP_01857650.1| glycosyl transferase, group 2 family [*Planctomyces maris* DSM 8797]	2.00E+00	COG0463		Glycosyltransferases involved in cell wall biogenisis
Contig 11487	|XP_001258384.1| 1,3-beta-glucanosyltransferase, putative [*Neosartorya fischeri* NRRL 181]	4.00E+00	cl06842		glycosyl hydrolase family 17
	|EDP49809.1| 1,3-beta-glucanosyltransferase, putative [*Aspergillus fumigatus* A1163]	8.00E+00	cl12144		glycosyl hydrolase family 5
Contig 11792	|XP_002999868.1| cellulose synthase catalytic subunit [*Verticillium albo-atrum* VaMs.102]	1.00E+01	cd06421	DXD	CESA_CelA like cellulose synthase GT-2
			PRK11204	QXXRW	N-glycsoyl transferase
	|XP_001259116.1| glycosyl transferase, putative [*Neosartorya fischeri* NRRL 181]	1.00E+01	cd06421	DXD	CESA_CelA like cellulose synthase GT-2
	|XP_001273558.1| glycosyl transferase, putative [*Aspergillus clavatus* NRRL 1]	1.00E+01	cd06421	DXD	CESA_CelA like cellulose synthase GT-2
	|XP_748682.1| glycosyl transferase [*Aspergillus fumigatus* Af293]	1.00E+01	cd06421	DXD	CESA_CelA like cellulose synthase GT-2
Contig 12312	|YP_003695065.1| Cellulose synthase BcsB [*Starkeya novella* DSM 506]	6.00E-01	PRK11114		cellulose synthase regulator protein
Contig 12738	|YP_003637451.1| glycosyl transferase family 2 [*Cellulomonas flavigena* DSM 20109]	7.00E-01	cd04186		GT_2_like_c; Subfamily of GT2 (GT-A) of unknown function.
Contig 14629	|CBJ28249.1| Cellulose synthase (UDP-forming), family GT2 [*Ectocarpus siliculosus*]	2.00E+00	cd06421	DXD	CESA_CelA like cellulose synthase GT-2
Contig 15267	|EFL91633.1| glycosyltransferase II [*Candidatus Regiella insecticola* LSR1]	9.00E-01	cd11394		Glyco_tranf_GTA_type; Glycosyltransferase family A (GT-A)
Contig 16157	|YP_003411251.1| glycosyl transferase family 2 [*Geodermatophilus obscurus* DSM 43160]	2.00E-01	cd04186		GT_2_like_c; Subfamily of GT2 (GT-A) of unknown function.
Contig 17137	|ZP_06766496.1| glycosyltransferase, group 2 family [*Bacteroides xylanisolvens*]	1.00E+00	cd00761		Glyco_tranf_GTA_type; Glycosyltransferase family A (GT-A)
Contig 17339	|YP_001419299.1| putative beta (1-6) glucans synthase [*Xanthobacter autotrophicus* Py2]	3.20E+00	cl02191		Glyco_hydro_17; Glycosyl hydrolases family 17
Contig 17999	|YP_001112741.1| glycosyl transferase family protein [*Desulfotomaculum reducens* MI-1]	2.00E+00	cd02511		UDP-glucose beta 1,4 Glycosyltransferase
Contig 19222	|YP_003130477.1| glycosyl transferase family 2 [*Halorhabdus utahensis* DSM 12940]	9.10E+00	cd06423		CESA_like; CESA_like is the cellulose synthase superfamily
Contig 23477	|YP_003576339.1| family 2 glycosyl transferase [*Rhodobacter capsulatus* SB 1003]	3.70E-01	cd11394		Glyco_tranf_GTA_type; Glycosyltransferase family A (GT-A)
Contig 24032	|YP_003072318.1| glycosyltransferase, group 2 family [*Teredinibacter turnerae* T7901]	7.00E+00	cd06420		GT2 GalNAc transferase
Contig 27271	|YP_003239098.1| glycosyl transferase family 2 [*Ammonifex degensii* KC4]	8.00E-01	cd04186		GT_2_like_c; Subfamily of GT2 (GT-A) of unknown function.
			cd11394		Glyco_tranf_GTA_type; Glycosyltransferase family A (GT-A)

Contig 3502

|XP_002793109.1|1,3-β-glucanosyltransferase gel2 [*Paracoccidioides brasiliensis* Pb01]
Length=471, Score = 33.5 bits (75), Expect = 9.2, Identities = 16/53 (30%), Positives = 28/53 (52%), Gaps = 0/53 (0%) Frame = +2

```
Query  233  VQSYFLNIPRIFHLNVQLKRSMYISFFNGSGSFTATAIVTPAAHRLLPRTESA  391
            +Q   +N  RI++LN      + +S FN +G +    + +P AH+ L R + A
Sbjct  77   LQRLGVNTIRIYNLNPATNHDLCVSIFNAAGIYLILDVNSPIAHQSLNRADPA  129
```

Contig 10917

|ZP_01857650.1| glycosyl transferase, group 2 family protein [*Planctomyces maris* DSM 8797]
Length=304, Score = 37.0 bits (84), Expect = 2E+00, Identities = 15/26 (58), Positives = 22/26 (85), Gaps = 1/26 (4), Frame = +3

```
Query  27   ILGQFWAKKTR-KPSKIFFAQNHDHD  101
            +L +FWA+K R +PSKIFF++ +DHD
Sbjct  271  VLARFWAQKLRVRPSKIFFSKKNDHD  296
```

Contig 11487

|XP_001258384.1| 1,3-beta-glucanosyltransferase, putative [*Neosartorya fischeri* NRRL 181]
Length=540, Score = 38.5 bits (88), Expect = 4E00 Identities = 34/131 (26), Positives = 55/131 (42), Gaps = 23/131 (18), Frame = +2

```
Query  1685  LQFSDCSMYPRVSPTPGREGTKTSPAFCSIDASTPIQRLALRSTAGPSGEEFSGVLHACI  1864
             + F + + Y  VS    +  T+   ++ S       Q+LA  + +G    E++
Sbjct  292   MYFQEANNYGLVSIDGDKVSTRADYSYLS-------QQLASATPSGTKKSEYT-------  337

Query  1865  LRGQSCFPSQSPLIWCPPLPRPWSATEDPLPPVASPTVLHLIFASLNCG-GDTEKYLQI-  2038
                    P+ S L  CPP+   W AT  PLPP  +  +   +  SL+C   DT    Q+
Sbjct  338   -------PTNSALQSCPPVNGDWLATASPLPPSPNGDLCSCMEDSLSCALKDTISDDQVE  389

Query  2039  LVLGDRCGIDI  2068
              + G  CG D+
Sbjct  391   KLFGTVCGYDV  401
```

Contig 11792

|XP_002999868.1| cellulose synthase catalytic subunit [*Verticillium albo-atrum* VaMs.102] Length=777
|XP_001259116.1| glycosyl transferase, putative [*Neosartorya fischeri* NRRL 181] Length=738
|XP_001273558.1| glycosyl transferase, putative [*Aspergillus clavatus* NRRL 1] Length=739
Score = 34.3 bits (77), Expect = 1E01 Identities = 16/28 (57), Positives = 16/28 (57), Gaps = 0/28 (0), Frame = +3

```
Query  285  LGKFWVTALAPTPFAFWLVPEDFGGKTK  368
            LGK W TA    P  F  VPEDFGG  K
Sbjct  417  LGKGWKTAYIHEPLQFGTVPEDFGGHLK  444
```

Contig 12738

|YP_003637451.1| glycosyl transferase family 2 [*Cellulomonas flavigena* DSM 20109]
Length=335 Score = 39.3 bits (90), Expect = 7E-01, Identities = 25/45 (56), Positives = 27/45, (60), Gaps = 5/45 (11), Frame = -1

```
Query  725  RSGAG---DWVAVAGVEPTVGDVEGFLGGESAELLREEGFRARGG  591
            R GAG   DW+AV G +   GDV GF GG  A LLR E  RA GG
Sbjct  147  RWGAGVDRDWLAVDGTQSPPGDVFGFHGG--AALLRTEAVRAVGG  189
```

Contig 24032

>ref|YP_003072318.1| glycosyltransferase, group 2 family protein [*Teredinibacter turnerae*]
Length=272, Score = 33.9 bits (76), Expect = 7E00 Identities = 13/15 (87), Positives = 13/15 (87), Gaps = 0/15 (0), Frame = +1

```
Query  151  NGHNPSCWKADRLTV  195
            NGHN SCWKAD LTV
Sbjct  180  NGHNASCWKADALTV  194
```

Figure 11: Contig queries from the sequenced genome with partial identity to glycosyl transferases.

MKKNHGYEEEVEIDVLQTPQHLHHYHNNSVPQPYGGYGQPAPEYSSRPGTANGRQPQANPDEYYSVWEPAGTG
RYSAQSFGESPVHSRPETPSLNSAYGTPYDLRPPPKNVYSSDSGPTTPMQQSQLSLAALLPNGPAPVIDADWV
NKSNSIARLHDRDDADIWKGWKRYVFKFVPVLTILNTAMYLLYLGYRIYCVVAAQKLRNTTYAQAWVFIGVEI
AVALPALMHNIWTMMAMKKRLRPKLRLTSNDDV**P**S**VD**VFVTCCGEEDDLVADTVRAACNL**D**Y**P**RDRFRVVVL**D**
DGKSEGLENAVLGMSQTYPNLV**Y**IA**R**PKIPGKPH**H**F**KAGNLN**YGLDAVHQLPGGAASSWPPWTLTWYIPERDW
LRAVLPHLLVDDKMALACPPQLFYNTP**D**S**D**PLAQSLDFFVHVIEPIKDALGVAWCTGSGYVVRREALDQIGNF
PLGSLAE**D**VATSTLM**LGK**G**W**K**TA**YIHE**P**LQ**F**GT**VPEDFGG**HL**KQ**RT**RW**AIGTVDTSFKLNFCLWGDKVREMSF
AQRFSGFLYAMLSLYTVLLTISMFAIPIILVMGKPLVAFATDDQFRWLIRACFAATISNRLCEFALFIPAGYH
TGQRGSRYQLWMAPYIALCIVRSFILPTWLGGQAQAFKPTGSLGSALNERDAHSRKNMMRRLWAILVNYMGLF
HLGFVYLTLVGVVLTSYRCFYLDTTVTDVLRCLVTHAFWPPLTFLFICSSLWTPVAYAIDPPTMPEREALLDR
DPKTGVAHPTRQRKKIAFGGQAAWFELEYTFTTAYTALIFAASFFLF

Figure 12: CESA_CelA like cellulose synthase GT-2 sequence from Verticillium albo-atrum. The bold single underligned amino acids are partially conserved among β-1,3-glycosyl transferases. The double-underligned QXXRW is the glycosyl transferase processive motif.

*NSIEENAE*TGETQQRRGPAAGRRRSGLPPTAQRERAGSAVLERASQPAVAERRAAVQRARDGQPSPSRSED
PRERLSVGGVQHAGVFKD*RD**LGK**FWVTALAPT**P**FA**F**WL**VPEDFGG**KT**K**VVPGFLGPSAIHPHCKKTVLNVPK
SLDA**Q**CG**RW**PALRPKRGPQGTVLNNKKLRSLLDRPKMWNQETRVSGRCFLRCLKKA*K

Figure 13: Contig 11792. The highlighted sequences share identity with the CESA_CelA like cellulose synthase from *V. albo-atrum* where the bold amino acids show complete identity.

4.3.2.1 Contig 11792

Contig 11792 shows identity to the partially conserved fungal cellulose synthase sequence VPEDFGGXXK of *Verticillium albo-atrum*, *Aspergillus clavatus* and *Neosartorya fisheri* (Figure 11). The VPEDFGGXXK sequence is followed immediately by QRTRWA (Figure 12), the highly conserved QXXRW β-glycosyl transferase processive motif. The *Verticillium albo-atrum* β-glycosyl transferase domain A consists of the the UDP-glucose binding DDG motif, the partially conserved KAG and highly conserved DXD motifs. Domain

B motifs include a single D amino acid motif and the QXXRW. Amino acids not highlighted but in bold are at least partially conserved among plant (Delmer, 1999), fungal (Ruiz-Herrera *et al*., 2002; Beauvais *et al*., 2001) and bacterial (Stasinopoulos *et al*., 1999) β-1,3-glucan synthases. The amino acid sequence of contig 11792 has the QCGRW motif 28 amino acids downstream in frame with the VPEDFGGXXK sequence (Figure 13). The highlighted sections in bold are the amino acids which share identity between the contig 11792 and the *V. albo-atrum* subject sequences.

The nucleotide sequence was BLAST searched against NCBI *Euglena* EST's, but no EST showed significant identity. The presence of stop codons in this sequence and the lack of other confirmable motifs are most likely due to incorrect assembly of the contig. A cellulose synthase CelA like gene product, producing a β-1,3/β-1,4- glucan, was recently identified in *Aspergillus* with some identity to plant and bacterial β-1,3- and β-1,4- glycosyl transferases while being absent from other fungi such as *S. cerevisiae* (de Groot *et al*., 2009). As neither *Euglena* (Pigon, 1947) or *Aspergillus* (de Groot *et al*., 2009) produce cellulose and presence of the QXXRW motif and the VPEDFGGXXK sequence suggest that at least part of the sequence on contig 11792 may code for a β-1,3-glycosyl transferase involved in paramylon synthesis.

4.3.2.2 Contig 11487

Contig 11487 showed partial identity to *Aspergillus fumigatus* and *Neosartorya fisheri* β-1,3-glucanosyl transferases. The nucleotide sequence of the entire contig is 2494bp but only a small section (highlighted) shows partial identity to the β-1,3-glycosyl transferase sequences (Figure. 11; Figure. 14, highlighted). The amino acids in bold share identity with subject β-1,3-glucan synthase sequences in the +2 (Figure 14) reading frame. Interestingly, the β-1,3-glycosyl transferase KAG motif is partially conserved in the + 3 reading frame (Figure 15). The underlined section represents the location of the motif while the bold amino acids indicate shared identity. The KAG motif is only partially conserved amongst β-1,3-glycosyl transferases (Ruiz-Herrera *et al*., 2002; Beauvais *et al*., 2001; Stasinopoulos *et al*., 1999), however the non-identical amino acids in the motif are substituted with amino acids of similar polarity and charge. It can be speculated that an incorrect assembly or changes in the amino acid sequence may have caused a frame shift in the contig. When contig 11487 was BLAST searched against the transcriptome for other contig which share nucleotide identity, two smaller contigs 10042 and 11670 were identified that showed similar nucleotide sequences (data not shown) but significant differences for the amino acid sequences based on substitutions and deletions indicating sequence misreads during assembly.

QWRLVALLSLSELPFPMALPLFWQNHLSARPTDATALATVLSQAAVNVPSTLVWPPVASALEECPPLGEQP*A
AELRAAMGLAPNQQGNFQYCGLPASLQSSGSGMDVFPNGLTVRIHLPPPLIFLWPSLFAVVSAFFATDAFADR
PRG*AVYV*DALC*GSLCGMAEAPG*GI*WERPHWQSAVASITKCECSGGFLRDDPISHAPLHRLWRGGSCLA
WAGSGPKRPRLSPQQPHREPYNGGPVYLLEALSPC*LSASPSTGRPAGLLGPLSPLGALSHLPRHGRRVRTPL
PPREQSIRSPQPRPAVLRWLCLSLCPRRPPLASI*IPTIAVACTAGKSEFATNVQEPHHSSQLVLSHVENLFF
SLKGPACFQ*EANCSE*TFKEITKQNEVISGLHQTVGFCCLLVLQKCATYSMQL*RVDRDHSDTFV*RRHGAQ
VFHVAF*SGLGRPR*ESHSQPSRRLLSLDSQVRGLMANALSLISHRVRPKTRFMPPLFLVTLRPFSVVIRKGK
H*HPCRTPRICRGYLWTEGGSAGSPPRLPPET*VM*SVFFSVFNAQNCLCLQ**F**SDCSM**Y**PR**VS**PTPGREGTK**T**
SPAFC**S**IDASTPI**Q**R**LA**LRSTA**G**PSGE**E**FSGVLHACILRGQSCF**P**SQ**S**P**L**IW**CPP**LPRP**W**S**AT**ED**PLPP**VASP
TVLHLIFA**SL**N**C**GG**DT**EKYL**Q**ILVL**G**DR**CG**I**D**IMFLQEAGRPFFFHSFRVPSSSPAHHLEEEGGKQSWYEIPY
*GCVCAKTRCIAIFHSDGFGWCFGGCLTLPTSSDIYLGKHLHSPGCFHGAWKQKRACISDLLWLRPAPLMAMG
GFQRGSENSQATTPAVSCPWARGGMEFP

Figure 14: Contig 11487 ORF +3. Contig 11487 had partial identity to a putative β-1,3-glycosyltransferase of *N. fischeri* (Figure. 12). The highlighted section in bold are the amino acids sharing identity.

NGVWSHFFHYRSCPFQWPCRCSGKIIYRQGPPTPQPWRLSCHRQL*TCHLRWSGLLWHPLWRNAHLWENNPER
QSSGLRWV*PQINRATFSIAACRPRCSPLVLAWTFFRMASQ*GFIYHLPSFFYGLLFLP*CQHFLRPMHLLID
QEARLFMSKMLYVRVPSAAWQKRLGEASDGSVPIGKALLPRSPNVSVVGVF*GMTPSAMLPSTASGVVGAA*H
GQALDPSVLD*ALSSPIASRTMGDRFTSSRHCPPADFPRARALGDQRDFSARCLPSALFLTYPGTGAGFELHC
LLASSRFVPPSHGLRYCVGSAFPCAPADRPWRA**Y**ESQQSQS**HA**RP**GNLN**LPRTFKNPTILPNLFFLTWRICSF
LSRVPHAFSERLTVLNEHLRKLPSKMKSFQVSIKPLGSAACWFCKNVQHIPCNCSEWTAIIQTPLCNADMGPR
YFMWLFEVVWAGHVESHIRNPPAACSLWTAKSVV*WQMLFHSSVTGCVPKPVSCRPCFWLPSGHFLL*SGKGS
IDIRAAPHAFAVDTFGRREVQLDHPRDCRPKPE*CNLFFFRYSMHKIVYVYNFLTVLCIRGSAPRLGEKAPKR
RLHFVASMPQLLSND*PSDLQPDLLGRNFQGSSTHAF*EVNHASRPSHL*YGVLHCHAHGQPRKTHSLLLHPQ
LFSI*FLPV*IVAVTQKNICRF*CLEIDAV*TLCSSKRPDVHFFFTASEYQVLLLHTIWKRRGGSNPGTKFHI
EVVCVQKPGASQFSTVMGLDGVLVVVLHFPQAVTFILANIYIPQGVFMALGSKKGHAFLIFSG*GQHL*WQWG
DFNEEARTAKPPRRLYRARGPEGAWSS

Figure 15: Contig 11487 ORF +2. Contig 11487 in ORF +2 showed amino acids with identity (bold underligned) to the partially conserved β-1,3-glucan domain A KAG motif a frame shift may have been caused by incorrect assembly.

General discussion, conclusion and future work

To date, research on *Euglena* has focused mainly on phylogeny and the physical and morphological characteristics of the organism and its novel β-1,3-glucan storage polymer, paramylon. The most recent publication (Bäumer *et al*., 2001) identified two peptides, within the 670 kDa membrane-bound paramylon synthase complex, approximately 54 and 37 kDa with affinity for the substrate, UDP-glucose, as well as increased activity with Ca^{2+} as the co-factor similar to what has been observed in plant callose synthases. These results have been confirmed in our laboratory (van der Merwe, 2007). However, none of the genes involved in paramylon synthesis have been identified. In this study, several approaches were followed in an attempt to further identify genes involved in paramylon synthesis in *E. gracilis*.

Screening of a *E. gracilis* cDNA library using a polyclonal antibody and radio-labeled substrate did not identify any sequences encoding subunits of paramylon synthase, possibly due to the membrane-bound β-1,3-glycosyl transferases losing conformational stability when expressed in *E. coli*. Protein purification steps identified peptides associated to the paramylon membrane and involved in paramylon synthesis. In this study nine proteins were identified using a polyclonal antibody and the corresponding peptides were excised, digested and the sequences predicted by LCMSMS. The protein bands corresponded with those previously published (Bäumer *et al*., 2001). Five other peptides were also identified by the polyclonal antibody, possibly transport or structural proteins or β-1,3-glycosyl hydrolases associated to the membrane. Predicted peptides were used to probe a partially sequenced transcriptome sequenced in this study as well as an uncompleted genome from another study. As glycosyl transferase sequences are highly variable, only predicted peptides matching a highly conserved sequence or motif would be a useful probe. However, none of the predicted peptides identified putative glycosyl transferases from the genome or transcriptome.

The sequenced genome was screened using BLASTx for putative glycosyl transferase enzymes, specifically for glycosyl transferase GT-2 and β-1,3-glucan synthases. Candidate glycosyl transferases sharing specific domain structures and partial sequence identity to β-1,3-glucan synthase, cellulose synthase genes and glycosyl transferase GT-2 sequences were identified. Although all the listed sequences are potential candidates, two candidate sequences drew specific attention. A sequence on contig 11487 showed identity to *Neosartorya fisheri* and *Aspergillus fumigatus* β-1,3-glycosyl transferase. A KAG-like β-1,3-glycosyl transferase conserved motif lies upstream of the region showing identity to the β-1,3-glycosyl transferase (Stasinopoulos *et al*., 1999) but out of frame, likely due to missassembly of the contig. A sequence on contig 11792 showed similarity to a conserved cellulose synthase-like sequence of *Verticillium albo-atrum*, *Aspergillus fumigatus* and *Neosartorya fisheri*. Both the contig sequence and the subject sequences had a QXXRW motif (Delmer, 1999; Ruiz-Herrera *et al*., 2002; Beauvais *et al*., 2001), the processive motif, down-

stream and in-frame with the sequence showing identity. Interestingly, the query sequences showed identity to subject sequences of the same fungal genera. As these fungal genera do not synthesize cellulose but make a β-1,3-/β-,4- glucan from a celA cellulose synthase-like gene product of a similar molecular weight (de Groot *et al*., 2009), it can be speculated that the paramylon synthase genes may have similar sequence and structural characteristics.

Future work would include amplifying and cloning all candidate sequences from cDNA using primers based on the sequence information of the contigs. Cloned sequences would need to be expressed and screened for activity.

In conclusion, subunits of the paramylon synthase complex of *E. gracilis* were partially purified, unidentified proteins associated to the granular membrane were described and candidate β-1,3-glycosyl transferase neucleotide sequences were identified.

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Addendum A: Predicted peptide LCMSMS sequences of the purified paramylon synthase proteins.

Peptide sequence	NCBI Reference	E-value
95 kDa		
SLDSSGMK	ref|YP_001866300.1| putative glycosyl transferase [*Nostoc punctiforme* PCC 73102]	3018
APIALSPR	ref|YP_001557624.1| glycosyl transferase family protein [*Clostridium phytofermentans* ISDg]	1676
	ref|XP_001915260.1| PREDICTED: glycosyltransferase-like 1B [*Equus caballus*]	5434
DYAAGATR	ref|ZP_05280251.1| glycosyl transferase [*Bacteroides fragilis* 3_1_12]	9784
FAGKAAFL	ref|YP_755775.1| glycosyl transferase family protein [*Maricaulis maris* MCS10]	1676
YDAANDR	ref|YP_002785459.1| putative peptidoglycan glycosyltransferase, precursor [*Deinococcus deserti*]	3544
APIAALIR	ref|NP_681033.1| putative glycosyltransferase [*Thermosynechococcus elongatu*s BP-1]	1676
APLAALLR	ref|YP_191427.1| putative glycosyltransferase [*Gluconobacter oxydans* 621H]	287
	ref|ZP_04606480.1| glycosyl transferase family 2 [*Micromonospora* sp. ATCC 39149]	1676
VATVSLPR	ref|ZP_05059660.1| glycosyl transferase, group 2 family [*Verrucomicrobiae bacterium* DG1235]	7291
AVLSSLPR	ref|ZP_01879731.1| glucosyltransferase MdoH [*Roseovarius* sp. TM1035]	1676
GLEAALLR	ref|ZP_01302004.1| glucosyltransferase MdoH [*Sphingomonas* sp. SKA58]	1676
GSLLCPPR	ref|YP_002951700.1| putative glycosyltransferase [*Desulfovibrio magneticus* RS-1]	2249
	ref | NP 579090.1| glycosyl transferase [*Pyrococcus furiosus* DSM 3638]	2249
LDEQLRSR	ref|YP_003120742.1| glycosyl transferase group 1 [*Chitinophaga pinensis* DSM 2588]	517
	emb|CAD97418.1| putative transmembrane glycosyltransferase [*Branchiostoma floridae*]	517
EAADDDIVQALNQYTK	ref|YP_001684350.1| glycosyl transferase family protein [*Caulobacter* sp. K31]	146
DLAKQFSIPSDIEENNNFQK	ref|YP_003293302.1| putative glycosyltransferase [*Lactobacillus johnsonii* FI9785]	80
80kDA		
APIALSPR	ref|YP_001557624.1| glycosyl transferase family protein [*Clostridium phytofermentans* ISDg]	1678
	ref|XP_001915260.1| PREDICTED: glycosyltransferase-like 1B [*Equus caballus*]	5438
APIAALIR	ref|NP_681033.1| putative glycosyltransferase [*Thermosynechococcus elongatus* BP-1]	1678
APLAALLR	ref|YP_191427.1| putative glycosyltransferase [*Gluconobacter oxydans* 621H]	287
	ref|ZP_04606480.1| glycosyl transferase family 2 [*Micromonospora* sp. ATCC 39149]	1678
GSHAIAIR	ref|XP_002523261.1| UDP-glucosyltransferase, putative [*Ricinus communis*]	7296
LAPAALLR	ref|YP_001221275.1| putative glycosyl transferase [*Clavibacter michiganensis subsp. michiganensis*]	2251
LPAALALR	ref|YP_003265416.1| glycosyl transferase family 2 [*Haliangium ochraceum* DSM 14365]	2251
LADALSPR	ref|YP_002480849.1| glycosyl transferase group 1 [*Desulfovibrio desulfuricans* ATCC 27774]	1250
VATVSLPR	ref|ZP_05059660.1| glycosyl transferase, group 2 family protein [*Verrucomicrobiae bacterium* DG1235]	7291
VAERDPR	ref|YP_003182395.1| glycosyl transferase family 2 [*Eggerthella lenta* DSM 2243]	3544
	ref|ZP_04055146.1| glycosyl transferase, family 2 [*Porphyromonas uenonis* 60-3]	3544
	ref|YP_001877371.1| glycosyl transferase family 2 [*Akkermansia muciniphila* ATCC BAA-835]	3544
	ref|YP_001611499.1| glycosyltransferase [*Sorangium cellulosum* 'So ce 56']	3544
VEAAISPR	ref|YP_001241524.1| putative glycosyl transferase, group 1 [*Bradyrhizobium* sp. BTAi1]	1676

EAVAAEPR	ref|ZP_05803199.1| glycosyl transferase family 2 [*Streptomyces flavogriseus* ATCC 3331]	1676
GLEAALLR	ref|ZP_05782651.1| putative glycosyl transferase [*Citreicella* sp. SE45]	1678
ISAVLPSR	ref|YP_645053.1| glycosyl transferase family protein [*Rubrobacter xylanophilus* DSM 9941]	1249
ISAVILVK	ref|NP_908016.1| glycosyltransferase [*Wolinella succinogenes* DSM 1740]	214
	ref|ZP_04632793.1| Glycosyl transferase, group 2 family protein [*Yersinia frederiksenii* ATCC 33641]	2249
	ref|YP_566838.1| polysaccharide biosynthesis protein [*Methanococcoides burtonii* DSM 6242]	2249
	ref|XP_002379617.1| chitin synthase, putative [*Aspergillus flavus* NRRL3357]	3018
	ref|ZP_04808199.1| glycosyltransferase [*Helicobacter pullorum* MIT 98-5489]	4050
AEAVVVVR	ref|YP_002565735.1| polysaccharide biosynthesis protein [*Halorubrum lacusprofundi* ATCC 49239]	1250
EAAVLALR	ref|NP_001154712.1| ATGSL12; 1,3-beta-glucan synthase/transferase [*Arabidopsis thaliana*]	2251
SLAVEPAR	emb|CAI30069.1| glycosyltransferase [*Triticum aestivum*]	1250
LKDGPNMAK	ref|ZP_05625831.1| glycosyl transferase, group 1 [*Campylobacter gracilis* RM3268]	2532
	ref|ZP_04947215.1| Glycosyltransferase [*Burkholderia dolosa* AUO158]	2532
	ref|ZP_02952387.1| capsular polysaccharide biosynthesis protein [*Clostridium perfringens* D str.]	4559
ELSVNINGK	ref|ZP_05390096.1| glycosyl transferase family 2 [*Clostridium carboxidivorans* P7]	582
IEWNGNIK	ref|ZP_01550917.1|putative bifunctional glycosyltransferase, [*Stappia aggregata* IAM 12614]	932
TLLTLTSER	ref|YP_003327119.1| glycosyl transferase family 2 [*Xylanimonas cellulosilytica* DSM 15894]	1406
TIMIKSNVK	ref|YP_695362.1| glycosyl transferase, group 2 family protein [*Clostridium perfringens* ATCC 13124]	1406
	ref|NP_896548.1| glycosyltransferase [*Synechococcus* sp. WH 8102]	1406
	gb|ABV65023.1| glycosyltransferase [*Dorosoma cepedianum*]	2532
DVLVHPPTR	gb|EEQ85757.1| UDP-glucose:sterol glycosyltransferase [*Ajellomyces dermatitidis* ER-3]	180
	ref|XP_002629213.1| UDP-glucose:sterol glycosyltransferase [*Ajellomyces dermatitidis* SLH14081]	180
TLLDAFLIK	ref|YP_001839410.1| putative glycosyltransferase [*Leptospira biflexa serovar* Patoc strain 'Patoc 1]	582
DKEGEEGNR	dbj|BAF75879.1| glucosyltransferase [*Dianthus caryophyllus*]	2532
HLELPAPLR	ref|ZP_02363972.1| glycosyl transferase, group 1 family protein, putative [*Burkholderia oklahomensis*]	781
	ref|ZP_02926586.1| Glycosyl transferase, group 1 family protein [*Verrucomicrobium spinosum* DSM]	1048
	ref|NP_868749.1| polysaccharide biosynthesis protein [*Rhodopirellula baltica* SH 1]	1048
LDVYVAPRI	ref|ZP_03921336.1| possible group 1 glycosyl transferase [*Corynebacterium pseudogenitalium* ATCC]	582
	ref|YP_002760198.1| putative glycosyltransferase [*Gemmatimonas aurantiaca* T-27]	582
MRPAVDGGSR	ref|YP_003112418.1| Peptidoglycan glycosyltransferase [*Catenulispora acidiphila* DSM 44928]	2831
DVNGGGTSPEK	ref|YP_446025.1| cellulose-binding domain-containing protein [*Salinibacter ruber* DSM 13855]	2091
RTDGSFDLIR	ref|YP_003143811.1| glycosyl transferase [*Slackia heliotrinireducens* DSM 20476]	868
AILDQVKVYTK	ref|ZP_06061385.1| glycosyltransferase [*Streptococcus* sp. 2_1_36FAA]	267
SCWDNLSTNSR	ref|YP_001551180.1| cell wall biosynthesis glycosyltransferase [*Prochlorococcus marinus* str. MIT 9211]	645
HTSSFLHPSNR	ref|NP_193283.2| UGT84A1; UDP-glycosyltransferase/ sinapate 1-glucosyltransferase [*A. thaliana*]	645
AEDEWKAQVAR	ref|YP_002959931.1| Glycosyltransferase [*Thermococcus gammatolerans* EJ3]	480
MALITTISAPER	ref|YP_003179855.1| glycosyl transferase family 2 [*Atopobium parvulum* DSM 20469]	1553
EADIDRTLLTR	ref|ZP_05077132.1| glycosyl transferase, group 2 family protein [*Rhodobacterales bacterium* Y4I]	267
MLIPVMRWQR	ref|YP_001634862.1| glycosyl transferase group 1 [*Chloroflexus aurantiacus* J-10-fl]	482
VAGARFCAHIER	ref|YP_114970.1| glycosyl transferase, group 1 family protein [*Methylococcus capsulatus* str. Bath]	863
RAGAVVDFIPER	ref|YP_001234644.1| glycosyl transferase WecB/TagA/CpsF [*Acidiphilium cryptum* JF-5]	198
NINLEDEKINK	ref|ZP_04822410.1| glycosyltransferase [*Clostridium botulinum* E1 str.]	199
VQARFDQSHNK	ref|XP_001944335.1| PREDICTED: similar to glucosyl/glucuronosyl transferases [*Acyrthosiphon pisum*]	2805
ALMENGGLHEMR	ref|XP_002301856.1| cellulose synthase [*Populus trichocarpa*]	198
	gb|AAN28294.1| cellulose synthase 2 [*Gossypioides kirkii*]	198

	gb|AAN28291.1| cellulose synthase 2 [*Gossypium raimondii*]	198
	gb|AAN28290.1| cellulose synthase 2 [*Gossypium herbaceum*]	198
	gb|AAN28293.1| cellulose synthase 2 [*Gossypium barbadense*]	198
	gb|AAO15532.1|AF458083_1 cellulose synthase [*Arabidopsis thaliana*]	198
	gb|AAB37767.1| cellulose synthase [*Gossypium hirsutum*]	198
	dbj|BAB09063.1| cellulose synthase catalytic subunit-like protein [*Arabidopsis thaliana*]	198
	ref|NP_199216.2| CESA4; cellulose synthase/ transferase, [*Arabidopsis thaliana*]	198
ALYPEVAVAVVAR	ref|YP_001381596.1| glycosyl transferase group 1 [Anaero*myxobacter* sp. Fw109-5]	110
	ref|YP_383895.1| peptidoglycan glycosyltransferase [*Geobacter metallireducens* GS-15]	265
AGAAALTEKSWPR	ref|YP_001819422.1| glycosyl transferase group 1 [*Opitutus terrae* PB90-1]	147
	ref|YP_319626.1| glycosyl transferase family protein [*Nitrobacter winogradskyi* Nb-255	198
	ref|YP_002499497.1| glycosyl transferase group 1 [*Methylobacterium nodulans* ORS 2060]	478
QKTPAFDYFIK	ref|ZP_04778607.1| family 2 glycosyltransferase [*Sphingobacterium spiritivorum* ATCC 33861]	1161
	ref|YP_957580.1| glycosyl transferase family protein [*Marinobacter aquaeolei* VT8]	481
	ref|YP_001341511.1| glycosyl transferase family protein [*Marinomonas* sp. MWYL1]	481
QLRGVISSLEQK	ref|YP_669975.1| putative glycosyltransferase [*Escherichia coli* 536]	863
	ref|ZP_03031752.1| mannosyl transferase [*Escherichia coli* F11]	863
LASAMGAPGLAGDAR	ref|ZP_03450438.1| glycosyl transferase, group 2 family protein [*Burkholderia pseudomallei* 576]	264
	ref|ZP_04889698.1| glycosyl transferase, group 2 family protein [*Burkholderia pseudomallei* 1655]	264
	ref|ZP_02509252.1| putative cellulose synthase [*Burkholderia pseudomallei* BCC215]	264
	ref|YP_337461.1| beta 1,3 glucan synthase catalytic subunit [*Burkholderia pseudomallei* 1710b]	264
TGREGTTGYSSNK	gb|AAN01252.1| Unknown protein similar to putative cellulose synthase [*Oryza sativa*]	0.007
	gb|ABB47240.2| Cellulose synthase family protein, expressed [*Oryza sativa* (japonica cultivar-group)]	0.007
	ref|XP_567874.1| beta-1,3 glucan biosynthesis-related protein [*Cryptococcus neoformans*]	2077
YEFLQNRECMK	ref|ZP_06086789.1| glycosyltransferase family 2 [*Bacteroides* sp. 3_1_33FAA]	358
HASTGDLTTIQDTK	ref|YP_002430018.1| glycosyl transferase family 2 [*Desulfatibacillum alkenivorans* AK-01]	858
	ref|ZP_05922740.1| glycosyl transferase, family 2 [*Enterococcus faecium* TC 6]	1151

72 kDa

LLGSRGFNAVSR	ref|YP_168098.1| glucosyltransferase MdoH [*Ruegeria pomeroyi* DSS-3]	1562
	gb|ABC25314.1| glucans biosynthesis glucosyltransferase H [uncultured marine bacterium Ant24C4]	2096
	ref|ZP_05122270.1| glucans biosynthesis glucosyltransferase H [*Rhodobacteraceae bacterium* KLH11]	0.071
	ref|ZP_01055929.1| glucosyltransferase MdoH [*Roseobacter* sp. MED193]	268
	ref|YP_002550848.1| glucosyltransferase [*Agrobacterium vitis* S4]	868
	ref|ZP_05787035.1| glucans biosynthesis glucosyltransferase H [*Silicibacter lacuscaerulensis* ITI-1157]	1164
	ref|YP_001262990.1| polysaccharide biosynthesis protein [*Sphingomonas wittichii* RW1]	1164

62 kDa

MALITTISAPER	ref|YP_003179855.1| glycosyl transferase family 2 [*Atopobium parvulum* DSM 20469]	1154
ISMIEGGLVDLR	ref|ZP_05594618.1| polysaccharide biosynthesis protein [*Enterococcus faecalis* AR01/DG]	266
	ref|ZP_03950093.1| PST family polysaccharide transporter [*Enterococcus faecalis* TX0104]	266

IVCDLKVGDNVK	ref|YP_001353521.1| glucan biosynthesis protein D [*Janthinobacterium* sp. Marseille]	357
	ref|YP_001918677.1| nucleotide sugar dehydrogenase [*Natranaerobius thermophilus* JW/NM-WN-LF]	863
LSVEEMRGLIR	ref|ZP_02931446.1| glycosyltransferase [*Verrucomicrobium spinosum* DSM 4136]	267
AQASVSQTQNLR	ref|NP_200715.2| glycosyl transferase family 1 protein [*Arabidopsis thaliana*]	863
EENVNVHDVGNDNTIK	ref|YP_003307971.1| Peptidoglycan glycosyltransferase [*Sebaldella termitidis* ATCC 33386]	109
MNVGIVVHGPEIIDSGFAEK	ref|YP_002938628.1| Glycosyltransferase Family 2 modular protein [*Eubacterium rectale* ATCC 33656]	194
QYGSVANYLEKGIGITAAEK	ref|ZP_06026371.1| glycosyl transferase group 1 [*Fusobacterium periodonticum* ATCC 33693]	144
ISSHSLEELAAELQISKEK	ref|YP_001450314.1| glycosyltransferase [*Streptococcus gordonii str. Challis* substr. CH1]	14

60 kDa		
-		
ICSEGAKIL	ref|XP_001845522.1| glucosyl/glucuronosyl transferase [*Culex quinquefasciatus*]	1409
	ref|XP_001663166.1| glucosyl/glucuronosyl transferases [*Aedes aegypti*]	2537
MIKQQSAK	ref|XP_001827919.1| nucleotide-sugar transporter [*Enterocytozoon bieneusi* H348]	2255
QLGDYIPK	ref|ZP_03131719.1| glycosyl transferase group 1 [*Chthoniobacter flavus* Ellin428]	3026
MIADVVSAK	ref|YP_949787.1| putative glycosyl transferase, group 1 family protein [*Arthrobacter aurescens* TC1]	2537
FTRDWTK	ref|YP_001404698.1| glycosyl transferase, group 1 [*Candidatus Methanoregula boonei* 6A8]	1970
FTRSGTER	ref|YP_002753948.1| glycosyl transferase, group 2 family [*Acidobacterium capsulatum* ATCC 51196]	1250
TFRMQDR	ref|YP_460432.1| glycosyltransferase [*Syntrophus aciditrophicus* SB]	2643
KIAHSLER	dbj|BAB86933.1| glucosyltransferase like protein [*Vigna angularis*]	932
AAAATGAAGHR	ref|YP_002230518.1| cellulose synthase regulator protein [*Burkholderia cenocepacia* J2315]	1561
	emb|CAR51686.1| cyclic di-GMP binding protein [*Burkholderia cenocepacia* J2315]	1561
LQAAARPAR	ref|ZP_02204800.1| glycosyl transferase, group 1 [*Dehalococcoides* sp. VS]	324
	ref|YP_336071.1| putative glycosyltransferase [*Burkholderia pseudomallei* 1710b]	1891
LQAANIPVK	ref|YP_001279140.1| glycosyl transferase, group 1 [*Psychrobacter* sp. PRwf-1]	1050
LSLLLSQKDSLIK	ref|ZP_03560622.1| polysaccharide biosynthesis protein [*Glaciecola sp*. HTCC2999]	265
AMMDLAVPVLQADLK	ref|YP_001748263.1| glycosyl transferase group 1 [*Pseudomonas putida* W619]	147
DSILLYYVTRMLK	gb|ABP96910.1| cellulose synthase 1 [*Phytophthora ramorum*]	147
QVNSIYGKFSASWK	ref|YP_003182856.1| glycosyl transferase family 51 [*Eggerthella lenta* DSM 2243]	1151
SDLDAALSELSVALGEVQR	ref|YP_002128859.1| glycosyltransferase [*Phenylobacterium zucineum* HLK1]	10
GVCIKTINQGLTFSYSNK	ref|ZP_05493841.1| glycosyltransferase 36 [*Clostridium papyrosolvens* DSM 2782]	472
CAAVPTTSDFHTEMAQYVFTMNRVGK	ref|YP_622459.1| putative ABC transporter ATP-binding protein [*Burkholderia cenocepacia* AU 1054]	4.00E-17
RNSGWVFENPSIGVLELWVLATNFR	ref|XP_001930359.1| glucan 1,3-beta-glucosidase [*Pyrenophora tritici-repentis* Pt-1C-BFP]	44
KAAVVANVYADAAGDLNTVFDNLPTINK	ref|ZP_05649292.1| glycosyltransferase [*Enterococcus gallinarum* EG2]	7.5
	ref|NP_948694.1| putative exopolysaccharide polymerization protein [*Rhodopseudomonas palustris*]	44

50 kDa		
CMSSHI	ref|ZP_02926473.1| glycosyl transferase, group 1 family protein [*Verrucomicrobium spinosum*]	9905
EHAAHL	ref|YP_002129322.1| glycosyl transferase, group 2 family protein [*Phenylobacterium zucineum* HLK1]	4100
MHCMR	ref|ZP_05476892.1| glycosyltransferase probably involved in cell wall biogenesis [*Streptomyces sp*. AA4]	11076
	emb|CBH17274.1| endosomal integral membrane protein, putative [*Trypanosoma brucei gambiense*]	11076
MHDFK	ref|ZP_03967889.1| possible glycosyltransferase [*Sphingobacterium spiritivorum* ATCC 33300]	4585

MHFDK	ref|XP_002174225.1| cell wall alpha-1,3-glucan synthase mok12 [*Schizosaccharomyces japonicus* yFS275]	4585
GHGGGRQR	ref|YP_003324970.1| glycosyl transferase group 1 [*Xylanimonas cellulosilytica* DSM 15894]	9836
HNGGVGRK	gb|EEH54761.1| glycosyltransferase family 1 protein [*Micromonas pusilla* CCMP1545]	13198
GHGGGGARR	ref|NP_001151784.1| LOC100285419 [*Zea mays*] ; gb|ACG44302.1| glycosyl transferase	785
GPPGDIIR	ref|ZP_01463583.1| glycosyl transferase, group 1 family protein [*Stigmatella aurantiaca* DW4/3-1]	2262
GSHAIAIR	ref|XP_002523261.1| UDP-glucosyltransferase, putative [*Ricinus communis*]	7336
IGEAALLR	ref|ZP_04659432.1| family 2 glycosyl transferase [*Selenomonas flueggei* ATCC 43531]	2255
	ref|ZP_00047747.2| COG2943: Membrane glycosyltransferase [*Magnetospirillum magnetotacticum*]	2255
VATVSLPR	ref|ZP_05059660.1| glycosyl transferase, group 2 family protein [*Verrucomicrobiae bacterium* DG1235]	7311
AVLSSLPR	ref|ZP_01879731.1| glucosyltransferase MdoH [*Roseovarius* sp. TM1035]	1681
IGEAAPER	ref|ZP_03129620.1| glycosyl transferase group 1 [*Chthoniobacter flavus* Ellin428]	4061
EAVAAEPR	ref|ZP_05803199.1| glycosyl transferase family 2 [*Streptomyces flavogriseus* ATCC 33331]	1685
	ref|YP_003290600.1| glycosyl transferase group 1 [*Rhodothermus marinus* DSM 4252]	1685
IGEAAPER	ref|ZP_03129620.1| glycosyl transferase group 1 [*Chthoniobacter flavus* Ellin428]	4072
ISLGAEPR	ref|YP_001236071.1| glycosyl transferase, group 1 [*Acidiphilium cryptum* JF-5]	5463
IGEAALLR	ref|ZP_04659432.1| family 2 glycosyl transferase [*Selenomonas flueggei* ATCC 43531]	2257
	ref|ZP_00047747.2| COG2943: Membrane glycosyltransferase [*Magnetospirillum magnetotacticum*]	2257
AEGIAEPR	ref|ZP_01460876.1| glycosyl transferase, group 2 family protein [*Stigmatella aurantiaca* DW4/3-1]	5463
XLVASIPR	gb|EEU48017.1| glycosyltransferase family 4 [*Nectria haematococca* mpVI 77-13-4]	5463
ELGAAEPR	ref|ZP_01048515.1| probable glycosyl transferase [*Nitrobacter* sp. Nb-311A]	5463
SLGLAEPR	ref|XP_001691334.1| glycosyltransferase-like protein [*Chlamydomonas reinhardtii*]	2262
AEAVVVVR	ref|YP_002565735.1| polysaccharide biosynthesis protein [*Halorubrum lacusprofundi* ATCC 49239]	1257
ASLVVVVR	gb|ABF99599.1| glycosyl transferase, group 2 family protein, expressed [*Oryza sativa*]	3037
DRVVPTR	ref|ZP_03971206.1| glycosyltransferase [*Corynebacterium glucuronolyticum* ATCC 51866]	4784
DRVVVVR	ref|XP_001325769.1| glycosyl transferase [*Trichomonas vaginalis* G3]	2657
IGEAAPER	ref|ZP_03129620.1| glycosyl transferase group 1 [*Chthoniobacter flavus* Ellin428]	4075
KSEIIGPV	ref|YP_002362773.1| glycosyl transferase group 1 [*Methylocella silvestris* BL2]	5467
VATVSLPR	ref|ZP_05059660.1| glycosyl transferase, group 2 family protein [*Verrucomicrobiae bacterium* DG1235]	7311
VAISALIR	ref|YP_002950715.1| polysaccharide biosynthesis protein [*Geobacillus* sp. WCH70]	2255
VAISLPSR	ref|YP_002158555.1| cellulose synthase operon protein YhjU [*Vibrio fischeri* MJ11]	2255
	ref|ZP_03154586.1| glycosyl transferase family 9 [*Cyanothece sp*. PCC 7822]	5448
VATVSLPR	ref|ZP_05059660.1| glycosyl transferase, group 2 family protein [*Verrucomicrobiae bacterium* DG1235]	7315
QLVLVSPS	ref|ZP_03888526.1| PMT family glycosyltransferase [*Geodermatophilus obscurus*]	1253
VAAEPLSR	ref|YP_743172.1| glycosyl transferase family protein [*Alkalilimnicola ehrlichii* MLHE-1]	4063
VAEALVLK	ref|ZP_04054840.1| glycosyl transferase, group 1 family protein [*Porphyromonas uenonis* 60-3]	2257
DGKPRAAK	ref|XP_001939736.1| GPI-anchored cell wall beta-1,3-endoglucanase EglC [*Pyrenophora tritici-repentis*]	7336
TAQRPNR	ref|YP_002469728.1| glycosyl transferase family 2 protein [*Bifidobacterium animalis subsp*. lactis]	4784
ANYDAILR	ref|YP_345320.1| glycosyl transferase family protein [*Rhodobacter sphaeroides* 2.4.1]	289
LIACADSDK	ref|ZP_01902080.1| glycosyltransferase involved in cell wall biogenesis [*Roseobacter* sp. AzwK-3b]	1054
NATNSVSSR	ref|ZP_04611996.1| Glycosyl transferase family 2 [*Yersinia rohdei* ATCC 43380]	2546
TVSLQAPSSR	gb|EEY57179.1| endo-1,3(4)-beta-glucanase, putative [*Phytophthora infestans* T30-4]	1567
HLELPAPLR	ref|NP_868749.1| polysaccharide biosynthesis protein [*Rhodopirellula baltica* SH 1]	1051
	ref|ZP_02363972.1| glycosyl transferase, group 1 family protein, putative [*Burkholderia oklahomensis*]	783
	ref|ZP_02926586.1| Glycosyl transferase, group 1 family protein [*Verrucomicrobium spinosum* DSM]	1051

YTELPNRLK	ref|YP_209926.1| putative glycosyltransferase [*Bacteroides fragilis* NCTC 9343]	242
AGMDASAIRNK	gb|AAN64562.1| glycosyltransferase [*Streptococcus gordonii*]	870
	ref|ZP_04062055.1| glycosyl transferase, group 1 family protein [*Streptococcus salivarius* SK126]	2102
	ref|YP_002951043.1| glycosyl transferase group 1 [*Geobacillus* sp. WCH70]	2102
AWHLPRILK	ref|XP_001300232.1| glycosyl transferase [*Trichomonas vaginalis* G3]	1054
ALAAALRIQGR	ref|ZP_04367828.1| glycosyl transferase [*Cellulomonas flavigena* DSM 20109]	200
AIKNIINIIK	ref|ZP_05633447.1| putative glycosyltransferase [*Fusobacterium ulcerans* ATCC 49185]	62
MGTTAALSQTTI	ref|YP_002280725.1| cellulose synthase subunit B [*Rhizobium leguminosarum* bv. trifolii WSM2304]	2096
GLYKSIVSATR	ref|YP_003067580.1| putative glycosyl transferase [*Methylobacterium extorquens* DM4]	2821
	ref|YP_001924069.1| glycosyl transferase group 1 [*Methylobacterium populi* BJ001]	2821
TYVKLVFSHK	ref|ZP_03154914.1| polysaccharide biosynthesis protein [*Cyanothece* sp. PCC 7822]	2829
ENENMSNQKK	ref|XP_001663167.1| glucosyl/glucuronosyl transferases [*Aedes aegypti*]	650
TYYFMIVER	ref|ZP_01127348.1| glucosyltransferase for synthesis of periplasmic glucans [*Nitrococcus mobilis*]	785
NRCLAELLGLK	ref|ZP_04441934.1| glycosyltransferase [*Lactobacillus rhamnosus* LMS2-1]	1164
	ref|ZP_03964155.1| group 1 glycosyl transferase [*Lactobacillus paracasei subsp. paracasei* ATCC 25302]	1164
	ref|YP_001611662.1| glycosyltransferase [*Sorangium cellulosum* 'So ce 56']	1164
ESEQINKEFTK	gb|ACA34492.1| putative glycosyltransferase [*Campylobacter jejuni*]	650
YLSYSYYCYK	ref|ZP_04054743.1| glycosyl transferase, group 2 family protein [*Porphyromonas uenonis* 60-3]	480
HKGLDTSVVSGPR	ref|ZP_01253348.1| glycosyl transferase, group 2 family protein [*Psychroflexus torquis* ATCC 700755]	1557
NDHAVGEDIRAR	ref|ZP_02488140.1| putative glucosyltransferase [*Burkholderia pseudomallei* NCTC 13177]	83
	ref|YP_002894939.1| hopanoid biosynthesis glycosyl transferase HpnI [*Burkholderia pseudomallei*]	83
	ref|ZP_02354145.1| glycosyl transferase, group 2 family protein [*Burkholderia oklahomensis* EO147]	83
	ref|ZP_02462016.1| glycosyl transferase, group 2 family protein [*Burkholderia thailandensis* MSMB43]	83
	ref|YP_104815.1| glycosyl transferase, group 2 family protein [*Burkholderia mallei* ATCC 23344]	83
AADGPTRFGTGFR	ref|YP_003006358.1| cellulose synthase operon C domain protein [*Dickeya zeae* Ech1591]	483
	ref|ZP_05102192.1| glycosyl transferase, group 1 [*Roseobacter* sp. GAI101]	1161
LTANANTENECKK	gb|ACJ63461.1| glucosyltransferase GTF8-2 [*Weissella confusa*]	645
APSPGPFVPPVSQR	ref|YP_925234.1| glycosyl transferase, group 1 [*Nocardioides* sp. JS614]	357
	ref|YP_001994131.1| glycosyl transferase family 2 [*Rhodopseudomonas palustris* TIE-1]	479
GIHSGTWKLSPPR	ref|YP_630159.1| polysaccharide biosynthesis/export protein [*Myxococcus xanthus* DK 1622]	481
SGFFSHCCLSFPSK	ref|ZP_05724441.1| glycosyl transferase group 1 [*Dickeya dadantii* Ech586]	863
	ref|ZP_06115144.1| glycosyl transferase, group 1 [*Clostridium hathewayi* DSM 13479]	1157
DAVAMFGYLSSDVANK	GENE ID: 374033 UGT8 | UDP glycosyltransferase 8 [*Gallus gallus*]	197
VKGNFVNGLPDGTLEK	ref|YP_679052.1| glucosyltransferase-S [*Cytophaga hutchinsonii* ATCC 33406]	197
	ref|ZP_04061534.1| glucosyltransferase-I [*Streptococcus salivarius* SK126]	147
WDYAVVASGGFFIAGK	ref|YP_152483.2| peptidoglycan synthetase [*Salmonella enterica subsp. enterica serovar Paratyphi* A]	476
VRIVCNSQLQADDVK	ref|YP_001126273.1| peptidoglycan binding protein [*Geobacillus thermodenitrificans* NG80-2]	356
	ref|ZP_04557557.1| glycosyl transferase [*Bacteroides* sp. D4]	478
	ref|YP_001735683.1| glycosyl transferase [*Synechococcus* sp. PCC 7002]	641
	ref|YP_283382.1| glycosyl transferase, group 1 [*Dechloromonas aromatica* RCB]	641
	ref|ZP_06060227.1| glycosyl transferase [*Streptococcus* sp. 2_1_36FAA]	860
LLAGFGNEVLAGYGIGSR	ref|YP_003201935.1| UDP-glucuronosyl/UDP-glucosyltransferase [*Nakamurella multipartita* DSM 44233]	60
GTGCSPDLPPPSRAEMGR	ref|YP_001537113.1| glycosyl transferase group 1 [*Salinispora arenicola* CNS-205]	60
	ref|YP_001158948.1| glycosyl transferase, group 1 [*Salinispora tropica* CNB-440]	60

	ref|YP_002481839.1| glycosyl transferase group 1 [*Cyanothece* sp. PCC 7425]	196
LITLWQAVVWRVTSR	ref|YP_002756046.1| glycosyl transferase, group 2 family [*Acidobacterium capsulatum* ATCC 51196]	265
AWNDEDPPDAFEEHR	ref|YP_001152946.1| glycosyl transferase family protein [*Pyrobaculum arsenaticum* DSM 13514]	19
	ref|NP_559585.1| glycosyl transferase, putative [*Pyrobaculum aerophilum* str. IM2]	61
AVGAHHVERTAVFVGDLVDR	ref|YP_002648411.1| Exopolysaccharide biosynthesis glycosyl transferase WcaL, [*Erwinia pyrifoliae*]	45
QYPGTMLIVSHDRYLLNK	ref|YP_002509151.1| ABC transporter related [*Halothermothrix orenii* H 168]	3.00E-09

35 kDA

VVPAAAK	ref|NP_558604.1| glycosyltransferase (type 1) [*Pyrobaculum aerophilum* str. IM2]	2700
HGATAAK	gb|ACD03254.1| UDP-glycosyltransferase UGT93B9 [*Avena strigosa*]	21140
QHTRN	ref|YP_003269085.1| Glycosyl transferase, family 3-like protein [*Haliangium ochraceum* DSM 14365]	15100
	ref|ZP_05855684.1| glycosyl transferase [*Blautia hansenii* DSM 20583]	15100
AIDALIAR	ref|YP_001749767.1| glycosyl transferase group 1 [*Pseudomonas putida* W619]	949
ALDALLAR	ref|YP_002952721.1| putative glycosyltransferase [*Desulfovibrio magneticus* RS-1]	293
ANNAILVK	ref|XP_001150762.1| UDP glycosyltransferase 1 family, polypeptide A6 isoform 1 [*Pan troglodytes*]	1274
DALALLAR	ref|YP_003156902.1| glycosyl transferase group 1 [*Desulfomicrobium baculatum* DSM 4028]	1709
VEAAISPR	ref|YP_001241524.1| putative glycosyl transferase, group 1 [*Bradyrhizobium* sp. BTAi1]	1703
	ref|YP_841154.1| cell wall biogenesis regulatory protein [*Ralstonia eutropha* H16]	946
VAKAQAVR	ref|YP_002974304.1| glycosyl transferase group 1 [*Rhizobium leguminosarum* bv. trifolii WSM1325]	3067
VATVSLPR	ref|ZP_05059660.1| glycosyl transferase, group 2 family protein [*Verrucomicrobiae bacterium* DG1235]	7408
VAERDPR	ref|YP_003182395.1| glycosyl transferase family 2 [*Eggerthella lenta* DSM 2243]	3600
	ref|ZP_04055146.1| glycosyl transferase, family 2 [*Porphyromonas uenonis* 60-3]	3600
	ref|YP_001877371.1| glycosyl transferase family 2 [*Akkermansia muciniphila* ATCC BAA-835]	3600
IGEAAPER	ref|ZP_03129620.1| glycosyl transferase group 1 [*Chthoniobacter flavus* Ellin428]	4115
IGEAAPER	ref|ZP_03129620.1| glycosyl transferase group 1 [*Chthoniobacter flavus* Ellin428]	4129
IGEAALLR	ref|ZP_04659432.1| family 2 glycosyl transferase [*Selenomonas flueggei* ATCC 43531]	2294
GLEAALLR	ref|ZP_05782651.1| putative glycosyl transferase [*Citreicella* sp. SE45]	1709
	ref|ZP_01302004.1| glucosyltransferase MdoH [*Sphingomonas* sp. SKA58]	1709
AVTVVEPK	ref|YP_317158.1| glycosyl transferase family protein [*Nitrobacter winogradskyi* Nb-255]	163
VAAVTPER	ref|ZP_04775004.1| glycosyl transferase family 2 [*Allochromatium vinosum* DSM 180]	3077
GGNAAALLR	ref|YP_003336149.1| Glycosyltransferase-like protein [*Streptosporangium roseum* DSM 43021]	2580
EAVAAEPR	ref|ZP_05803199.1| glycosyl transferase family 2 [*Streptomyces flavogriseus* ATCC 33331]	1703
	ref|YP_003290600.1| glycosyl transferase group 1 [*Rhodothermus marinus* DSM 4252]	1703
	ref|YP_966185.1| peptidoglycan glycosyltransferase [*Desulfovibrio vulgaris* subsp.	1703
ELGAAEPR	ref|ZP_01048515.1| probable glycosyl transferase [*Nitrobacter* sp. Nb-311A]	5521
IGEAAPER	ref|ZP_03129620.1| glycosyl transferase group 1 [*Chthoniobacter flavus* Ellin428]	4115
VEAAISPR	ref|YP_001241524.1| putative glycosyl transferase, group 1 [*Bradyrhizobium* sp. BTAi1]	1703
	ref|ZP_02371622.1| lipopolysaccharide biosynthesis protein [*Burkholderia thailandensis*]	705
	ref|YP_841154.1| cell wall biogenesis regulatory protein [*Ralstonia eutropha* H16]	946
LADALSPR	ref|YP_002480849.1| glycosyl transferase group 1 [*Desulfovibrio desulfuricans subsp. desulfuricans* str.]	1269
LDAALSPR	ref|ZP_01878060.1| glycosyl transferase, group 1 family protein [*Roseovarius* sp. TM1035]	1269
	ref|YP_110445.1| lipopolysaccharide biosynthesis protein [*Burkholderia pseudomallei*]	1269
LSSPATLNSR	ref|YP_003138348.1| Periplasmic component of Tol biopolymer transport system-like [*Cyanothece* sp.]	1570

LEAAADGITGK	ref|YP_685025.1| glycosyltransferase (group 1) [uncultured methanogenic archaeon RC-I]	1167
MRLGILGTGK	ref|YP_385123.1| glycosyltransferase-like protein [*Geobacter metallireducens* GS-15]	872
TWLAPDGTGK	gb|ABC67269.1| putative glycosyltransferase [*Streptomyces hygroscopicus* subsp.	1587
EVRIGSASVR	ref|YP_003013567.1| Peptidoglycan glycosyltransferase [*Paenibacillus sp*. JDR-2]	2108
EVVEAAGGSVR	ref|YP_003153748.1| predicted glycosyltransferase [*Brachybacterium faecium* DSM 4810]	200
EAILTGQSVR	ref|ZP_04832497.1| glycosyl transferase, group 1 family [*Prevotella melaninogenica* ATCC 25845]	887
SAVNGEAANIK	ref|ZP_05138412.1| glycosyl transferase, group 1 [*Prochlorococcus marinus* str. MIT 9202]	2866
	ref|ZP_02185159.1| glycosyl transferase, group 1 family protein [*Carnobacterium* sp. AT7]	1186
QKLPQIVIDK	ref|YP_684816.1| putative oligosaccharyltransferase [uncultured methanogenic archaeon RC-I]	661
TSKGSAYLNLK	ref|YP_684816.1| putative oligosaccharyltransferase [uncultured methanogenic archaeon RC-I]	2866
	ref|XP_002519441.1| UDP-glucosyltransferase, putative [*Ricinus communis*]	884
RVSALNIAHLK	gb|EES53993.1| putative glycosyl transferase, group 1 [*Leptospirillum ferrodiazotrophum*]	2136
GPSSPSSSADACR	ref|ZP_06276423.1| Protein involved in beta-1 3-glucan synthesis-like protein [*Streptomyces sp*. ACT-1]	488
QVNDQYVSLAK	ref|ZP_04493766.1| glycosyltransferase [*Spirosoma linguale* DSM 74	1592
SFASNTLRGVGR	ref|ZP_05402172.1| putative polysaccharide biosynthesis protein [*Clostridium difficile* QCD-23m63]	1183
DILGTYERVAK	ref|NP_227976.1| geranyltranstransferase [*Thermotoga maritima* MSB8]	1186
	ref|YP_001738822.1| polyprenyl synthetase [*Thermotoga* sp. RQ2]	1186
LHHMSEEILR	ref|YP_948600.1| putative glycosyl transferase, group 1 family protein [*Arthrobacter aurescens* TC1]	493
	ref|YP_002514796.1| glycosyl transferase, group 1 [*Thioalkalivibrio* sp. HL-EbGR7]	887
QHQSSHTSENK	ref|ZP_01621904.1| Glycosyl transferase, family 2 [*Lyngbya* sp. PCC 8106]	884
IKATDFMVAMR	ref|ZP_03700828.1| glycosyl transferase family 9 [*Flavobacteria bacterium* MS024-3C]	884
	ref|XP_002486496.1| exo-beta-1,3-glucanase, putative [*Talaromyces stipitatus* ATCC 10500]	884
RTGHVLVAASSGK	gb|AAH56559.1| Dolichyl-diphosphooligosaccharide-protein glycosyltransferase [*Danio rerio*]	1179
GLPAGEPEMIIR	ref|YP_003122797.1| glycosyl transferase family 2 [*Chitinophaga pinensis* DSM 2588]	657
	ref|YP_003087422.1| Cellulose synthase (UDP-forming) [*Dyadobacter fermentans* DSM 18053]	657
LQSVPLDELLR	ref|YP_001999413.1| glycosyl transferase group 1 [*Chlorobaculum parvum* NCIB 8327]	491
	ref|NP_001063684.1| Os09g0518000 [*Oryza sativa* (japonica cultivar-group)]	273
GAFHTVSLQKYSK	ref|ZP_03395721.1| glycosyl transferase, group 2 family protein [*Pseudomonas syringae* pv. tomato T1]	1578
DPICIAXSSCCGKR	emb|CBH11780.1| ABC transporter, putative [*Trypanosoma brucei gambiense* DAL972]	362
EYVAIKQVPYQK	GENE ID: 6172670 Exig_1766 | glycosyl transferase family protein[*Exiguobacterium sibiricum* 255-15]	271
QNEYFCFATKSK	emb|CAJ70866.1| similar to glycosyl transferase family 1 [*Candidatus Kuenenia stuttgartiensis*]	879
EDIINRLLSNCIR	ref|NP_812284.1| glycosyltransferase [*Bacteroides thetaiotaomicron* VPI-5482]	202
LMILGTPFAAPNWK	ref|ZP_05670075.1| glucosyltransferase [*Enterococcus faecium* 1,231,410]	363
	ref|ZP_03374705.1| glucan biosynthesis protein [*Salmonella enterica subsp. enterica serovar* Typhi str.]	487
ANLTASSAKVAHHHD	ref|ZP_01063294.1| predicted calcium-binding protein [*Vibrio* sp. MED222]	485
KGQGSYFNSGTWAR	ref|ZP_03074363.1| Peptidoglycan glycosyltransferase [*Lactobacillus reuteri* 100-23]	363
	ref|ZP_04869645.1| glycosyl transferase [*Helicobacter canadensis* MIT 98-5491]	1176
	gb|ABY41241.1| CP7 beta-1,3-1,4-glucanase [*Paenibacillus polymyxa*]	202
AADADTLQAYTEEVIGK	ref|XP_001936633.1| polysaccharide synthase [*Pyrenophora tritici-repentis* Pt-1C-BFP]	360
EYGVYYFSDNPEIAK	ref|YP_002544914.1| glycosyltransferase protein [*Agrobacterium radiobacter* K84]	270
	ref|ZP_04061326.1| glucosyltransferase-I [*Streptococcus salivarius* SK126]	150
RIGSSSDTSSDTSSSSSSSSDK	ref|YP_925858.1| glycosyl transferase family protein [*Nocardioides* sp. JS614]	69
	ref|ZP_03864096.1| glycosyl transferase [*Kribbella flavida* DSM 17836]	168
	ref|ZP_03864798.1| putative glucan synthasis protein [*Kribbella flavida* DSM 17836]	168

QVYNVIRQLFQIAMPFGSK	ref|ZP_05316312.1| glycosyl transferase family 2 [*Nitrosomonas* sp. AL212]	148
SIAFSVCAAQEALWRISGSSR	ref|YP_001537238.1| glycosyl transferase family protein [*Salinispora arenicola* CNS-205]	265
MPDHLGQPVSQAVAFPNDTSK	ref|ZP_04775708.1| glycosyl transferase group 1 [*Allochromatium vinosum* DSM 180]	265
LQRGSAPYLQFELVVYSLR	ref|ZP_04773562.1| glycosyl transferase group 1 [*Allochromatium vinosum* DSM 180]	25
NREQDHASDAYTALLSSVLQR	ref|XP_812757.1| glycosyl transferase [*Trypanosoma cruzi strain* CL Brener]	145
	ref|XP_805961.1| glycosyl transferase [*Trypanosoma cruzi strain* CL Brener]	145
GLGMLGWVFFPPREDLSGGIDR	ref|YP_003129966.1| glycosyl transferase group 1 [*Halorhabdus utahensis* DSM 12940]	61
	ref|ZP_03997417.1| glycosyltransferase [*Halogeometricum borinquense* DSM 11551]	61
	ref|YP_658229.1| LPS glycosyltransferase [*Haloquadratum walsbyi* DSM 16790]	61
IDSSNSTICEVSPLVSLNGENLK	emb|CAP39914.1| UDP-GlcNAc diphosphorylase [*Trypanosoma brucei brucei*]	81
	ref|XP_635927.1| UDP-N-acetylglucosamine pyrophosphorylase [*Dictyostelium discoideum*	2.00E-12