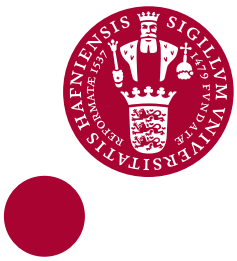

A diverse range of bacterial and eukaryotic chitinases hydrolyzes the LacNAc (Gal1-4GlcNAc) and LacdiNAc (GalNAc1-4GlcNAc) motifs found on vertebrate and insect cells

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A Diverse Range of Bacterial and Eukaryotic Chitinases Hydrolyzes the LacNAc (Galβ1–4GlcNAc) and LacdiNAc (GalNAcβ1–4GlcNAc) Motifs Found on Vertebrate and Insect Cells*[S]

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Background: The biological role(s) of chitinases other than hydrolysis/metabolism of chitin is currently not well understood.
Results: A *Salmonella* chitinase binds *N*-acetyllactosamine (LacNAc), a common component of mammalian glycoconjugates. Furthermore, five bacterial and eukaryotic chitinases hydrolyze terminal LacNAc and LacdiNAc from model substrates.
Conclusion: LacdiNAc and LacNAc- glycans are substrates for chitinases.
Significance: Vertebrate and invertebrate molecules carrying LacNAc and LacdiNAc motifs are potential chitinase targets.

There is emerging evidence that chitinases have additional functions beyond degrading environmental chitin, such as involvement in innate and acquired immune responses, tissue remodeling, fibrosis, and serving as virulence factors of bacterial pathogens. We have recently shown that both the human chitotriosidase and a chitinase from *Salmonella enterica* serovar Typhimurium hydrolyze LacNAc from Galβ1–4GlcNAcβ-tetramethylrhodamine (LacNAc-TMR (Galβ1–4GlcNAcβ(CH_2)$_8$-CONH(CH_2)$_2$NHCO-TMR)), a fluorescently labeled model substrate for glycans found in mammals. In this study we have examined the binding affinities of the *Salmonella* chitinase by carbohydrate microarray screening and found that it binds to a range of compounds, including five that contain LacNAc structures. We have further examined the hydrolytic specificity of this enzyme and chitinases from *Sodalis glossinidius* and *Polysphondylium pallidum*, which are phylogenetically related to the *Salmonella* chitinase, as well as unrelated chitinases from *Listeria monocytogenes* using the fluorescently labeled substrate analogs LacdiNAc-TMR (GalNAcβ1–4GlcNAcβ-TMR), LacNAc-TMR, and LacNAcβ1–6LacNAcβ-TMR. We found that all chitinases examined hydrolyzed LacdiNAc from the TMR aglycone to various degrees, whereas they were less active toward LacNAc-TMR conjugates. LacdiNAc is found in the mammalian glycome and is a common motif in invertebrate glycans. This substrate specificity was evident for chitinases of different phylogenetic origins. Three of the chitinases also hydrolyzed the β1–6 bond in LacNAcβ1–6LacNAcβ-TMR, an activity that is of potential importance in relation to mammalian glycans. The enzymatic affinities for these mammalian-like structures suggest additional functional roles of chitinases beyond chitin hydrolysis.

Chitinases are ubiquitous enzymes found in archaea, bacteria, fungi, plants, and animals. They hydrolyze chitin, the second most abundant polysaccharide in nature. Chitin is a linear polymer comprised of β1–4-linked *N*-acetyl-D-glucosamine (GlcNAc) units and serves as a structural polymer in crustacean shells, in the cell walls of fungi, and in the exoskeletons of arthropods. The biological roles of some chitinases are well established, such as in chitin cycling in the marine environment by *Vibrio cholerae* and other *Vibrio* spp. (1), in yeast cell division (2), and in cuticle turnover and nutrient digestion in arthropods (3). Many plant chitinases are induced upon microbial infection, and some have the potential to inhibit the growth of fungal pathogens, hence displaying a defensive role (4, 5). However, the biological functions of numerous chitinases are

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[S] This article contains supplemental Table 1 and Fig. 1.
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TABLE 1
Overview of chitinases used in this study

Protein	Gene	Species	Source
*St*ChiA	*STM0018*[a]	*Salmonella* Typhimurium LT2	This study
*St*ChiAΔCBM	*SL0018ΔCBM*[a] (1936→2097)	*Salmonella* Typhimurium SL1344	(14)
*St*ChiA E223Q	*STM0018*, G697C	*Salmonella* Typhimurium LT2	This study
*St*ChiA E223QΔCBM	*STM0018*, G697C, Δ*CBM* (1936→2097)	*Salmonella* Typhimurium LT2	This study
*Lm*ChiA	*lmo1883*	*L. monocytogenes* EGD-e	(21)
*Lm*ChiB	*lmo0105*	*L. monocytogenes* EGD-e	This study
*Lm*ChiBΔCBM	*lmo0105* Δ*CBM* (2130→2265)	*L. monocytogenes* EGD-e	This study
*Pp*Chitinase	*EFA83839*	*P. pallidum* PN500	This study
*Sg*ChiA	*SG1474*	*S. glossinidius* str. morsitans	This study
S. griseus chitinase	Unspecified	*S. griseus*	Sigma
T. viride chitinase[b]	Unspecified	*T. viride*	Sigma

[a] The *STM0018* and *SL0018* genes have identical nucleotide sequence.
[b] According to manufacturer, a mixture of two chitinases.

still unknown, especially in organisms that rarely come into contact with chitin.

One eukaryotic example is chitinases encoded by cellular slime molds, such as *Dictyostelium* spp. and *Polysphondylium* spp. These organisms utilize mainly bacteria as a food source, and consumption of chitin-containing yeast supports growth only poorly. There are also examples of plant chitinases that have defensive physiological roles that are attributed to ribosome inactivating activity (6, 7). Humans produce two active chitinases, chitotriosidase and acidic mammalian chitinase (AMCase), that can be linked to a variety of disorders including lysosomal storage disease, asthma, and neurological diseases (8–11). Finally, it is now emerging that for several bacterial pathogens and symbionts, chitinases may be important as virulence or symbiotic factors as much as, or rather than, being important for chitin metabolism. Examples include enzymes produced by *Legionella pneumophila*, *Listeria monocytogenes*, and possibly *Salmonella* Typhimurium (12–14). Understanding the exact role of chitinases in infectious processes is, however, limited by the fact that their target substrates in the host remain unidentified.

The gene expression of *Salmonella* Typhimurium chitinase, *St*ChiA, is up-regulated during infection of macrophage and epithelial cells (15, 16). We recently found that the catalytic domain of this enzyme (*St*ChiAΔCBM) cleaves *N*-acetyllactosamine (LacNAc,[5] (Galβ1–4GlcNAc)) from the tetramethylrhodamine (TMR, see Fig. 1) fluorescently labeled substrate analog LacNAc-TMR in addition to hydrolyzing chitin, chitooligosaccharides, and the chitin pseudo-substrate *p*NP-$(GlcNAc)_2$ (14). Hydrolysis occurs with retention of configuration and is consistent with the TMR-linker occupying the + subsites of the enzyme with the Gal and GlcNAc of LacNAc in the −2 and −1 subsites, respectively. This is analogous to the hydrolysis of *p*NP-$(GlcNAc)_2$, where *p*-nitrophenyl (*p*NP) occupies the +1 subsite and $(GlcNAc)_2$ is bound to the −2 and −1 subsites. Because *N*-acetyllactosamine is a common component of mammalian glycans, it constitutes a possible chitinase target. We have also shown that human chitotriosidase cleaves LacNAc and LacdiNAc (GalNAcβ1–4GlcNAc) from LacNAc-TMR and LacdiNAc-TMR, respectively, with the latter having a turnover comparable to that of *p*NP-chitotrioside (17).

In this study we have cloned and characterized chitinases, which are phylogenetically related to the *Salmonella* enzyme *St*ChiA, from the bacterial insect endosymbiont *Sodalis glossinidius* and the eukaryotic slime mold *Polysphondylium pallidum* (see Table 1 and Fig. 2). These three enzymes share the common characteristic that they lack a signal peptide but are still predicted to be secretory proteins (14).

For comparison we have included the phylogenetically unrelated chitinases *Lm*ChiA and *Lm*ChiB (18) from *L. monocytogenes* (see Table 1 and Fig. 2) and the commercially available chitinases from the actinobacterium *Streptomyces griseus* and the mold *Trichoderma viride.* The chitinases included in the present study all belong to the CAZy family 18 glycoside hydrolases, although it should be noted that the two commercial available chitinases from *T. viride* and *S. griseus* remain uncharacterized in this regard.

We observed that the *Salmonella* chitinase S*t*ChiA was able to bind to potential glycan targets in hosts by use of a carbohydrate microarray. In addition, we found that all enzymes surveyed (with one exception) hydrolyzed LacdiNAc from fluorescently labeled conjugates, whereas several of the enzymes hydrolyzed LacNAc from analogous conjugates that are substrate analogs of glycans found in mammals.

EXPERIMENTAL PROCEDURES

Phylogenetic Analysis—The amino acid sequences of *Salmonella* Typhimurium SL1344 *St*ChiA (SL0018), *S. glossinidius* str. morsitans *Sg*ChiA (SG1474), *P. pallidum Pp*chitinase (EFA83839), *L. monocytogenes Lm*ChiA (*lmo1883*), and *Lm*ChiB (*lmo0105*) (Table 1) were from the GenBank™ database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/). Protein domain predictions including determination of the glycosyl hydrolase family 18 (GH_18) domains were obtained from the SMART website. Signal peptide predictions were performed using SignalP 4.0 from the Centre for Biological Sequence Analysis, Biocentrum, Technical University of Denmark. GH_18 domains of sequences representative of bacterial chitinases in addition to representatives of eukaryotic chitinases were included in the phylogenetic anal-

[5] The abbreviations used are: LacNAc, *N*-acetyllactosamine,Galβ1–4GlcNAc; LacNAc-TMR, Galβ1–4GlcNAcβ$(CH_2)_8$CONH$(CH_2)_2$NHCO-TMR; TMR, tetramethylrhodamine; LacdiNAc-TMR, GalNAcβ1–4GlcNAcβ$(CH_2)_8$CONH$(CH_2)_2$-NHCO-TMR; Type I-TMR, Galβ1–3GlcNAcβ$(CH_2)_8$CONH$(CH_2)_2$NHCO-TMR; *p*NP-$(GlcNAc)_2$, Galβ1–4GlcNAcβ-*p*NP; LacNAcβ1–6LacNAcβ-TMR, Galβ1–4-GlcNAcβ1-6 Galβ1–4GlcNAcβ$(CH_2)_2$NHCO-TMR; *St*, *Salmonella* Typhimurium; *Pp*, *P. pallidum*; *Lm*, *L. monocytogenes*; *Sg*, *S. glossinidius*; *p*NP, *p*-nitrophenyl.

ysis. The phylogenetic tree (see Fig. 2) based on the corresponding GH_18 domains was constructed by the neighbor-joining method using ClustalX Version 1.81 (19) and the Mega version 4 (20) software packages with default settings.

Production and Purification of Chitinases—The nucleotide sequences of the chitinase genes SG1474 of *S. glossinidius* str. morsitans and EFA83839 of *P. pallidum* PN500 were codon-optimized for expression in *Escherichia coli*, and the genes were synthesized with an N-terminal His_6 tag flanked by NcoI/XhoI restriction sites (Epoch Biolabs, Missouri City, TX). The synthetic genes were cloned into pET15b expression vectors, which were transformed into chemically competent *E. coli* TOP10 cells (Invitrogen) for long term storage. For protein expression the vector carrying SG1474 was transformed into *E. coli* BL21(DE3) cells (Novagen, Darmstadt, Germany), whereas the vector carrying EFA83839 was transformed into *E. coli* BL21/TUNER™(DE3) cells (Novagen). 10 ng of pET-15b-SG1474 or EFA83839 were added to 50 μl of *E. coli* TOP10, *E. coli* BL21(DE3), or *E. coli* BL21/Tuner™(DE3) cells. The reaction mixture was incubated on ice for 30 min, heat-shocked at 42 °C for 30 s, and incubated on ice for an additional 2 min, then 200 μl of Luria broth (LB; Merck) was added. The reaction mixture was incubated at 37 °C for 60 min with shaking and plated on LB agar plates containing 100 μg/ml carbenicillin and incubated overnight at 37 °C. Transformants were re-streaked on carbenicillin-containing LB plates. The following day colonies were grown in LB and stored as glycerol stocks.

The *L. monocytogenes* EGDe *chiB* gene (*lmo0105*) and a truncated version of *chiB* (*lmo1883ΔCBM*) lacking the C-terminal carbohydrate binding domain (amino acids 711–755) were amplified through PCR with primers designed to exclude the respective signal peptide sequences but including a His_6 tag at the N terminus. The PCR products were cloned into the pET-46 Ek/LIC vector and introduced into *E. coli* BL21(DE3) according to the manufacturer's instructions (Novagen pET-46 E/LIC vector kit). The absence of mis- and nonsense mutations was confirmed by sequencing (Macrogen, Seoul, Korea). The *E. coli* BL21(DE3) strain used for expression of the *L. monocytogenes* chitinase, *Lm*ChiA (*lmo1883*), was from a previous study (21). An *E. coli* BL21(DE3) strain carrying a *Salmonella* chitinase gene without the chitin-binding domain (*SL0018ΔCBM*) and used for expression of *St*ChiAΔCBM was prepared in a previous study (14). Expression vectors for the production of *Salmonella* wild type chitinase, *St*ChiA, and the inactive enzyme variants *St*ChiA E223Q and *St*ChiA E223QΔCBM were purchased from Epoch life sciences Inc. (Missouri City, TX). In brief, *St*ChiA E223Q was obtained by substituting the active site glutamate with a glutamine residue by site-directed mutagenesis (E223Q, GAA→CAA), whereas ChiA E223QΔCBM was obtained by a further truncation from amino acid 646. The synthetic genes were cloned into NdeI/XhoI restriction sites of the pETb15 expression vector carrying a His-tag coding sequence. The resulting vectors was transformed into *E. coli* BL21(DE3) as previously described.

For protein purification, up to 1 liter of *E. coli* BL21(DE3) cells were grown at 30 °C to an A_{600} of 0.4 before induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside. After induction, growth was continued for 21 h at 30 °C. For improved recovery and purification of the *Polysphondylium* chitinase, *Pp*Chitinase, *E. coli* BL21/Tuner™(DE3) cells were grown at 37 °C to an A_{600} of 0.6 and placed on ice to cool before induction with 0.3 mM isopropyl 1-thio-β-D-galactopyranoside. After induction, growth was continued for 21 h at 22 °C. The *E. coli* BL21(DE3) and/or *E. coli* BL21/Tuner cells were harvested by centrifugation and resuspended in 10 volumes of loading buffer, 20 mM MOPS, pH 7.2, containing 0.5 M NaCl and 5 mM imidazole. The cells were disrupted using a Constant Systems cell disruptor at 4 °C with a pressure of 1.36 kilobars. The lysate was centrifuged at 4 °C for 1.5 h at 48,000 × *g*, and the filtered supernatant was applied to a 1- or 5-ml HisTrap HP column (GE Healthcare) with a flow rate of 1 or 5 ml/min, respectively. For the *Listeria* enzymes, 1–2-ml columns of nickel-nitrilotriacetic acid-agarose (Qiagen) were used with a flow rate of 1 ml/min. All columns were washed with 100 ml of loading buffer before being eluted with 30 ml of 100 mM MOPS, pH 7.8, 0.5 M NaCl, and 0.5 M imidazole. For the *Sodalis* chitinase *Sg*ChiA and the *Salmonella* *St*ChiAΔCBM, fractions containing enzyme were combined, the buffer was exchanged to loading buffer by dialysis, and the enzymes were rechromatographed on a 1-ml HisTrap column with an imidazole gradient from 5 to 250 mM imidazole to obtain ultrapure enzymes. For all enzymes, the fractions containing enzyme were dialyzed against 50 mM sodium phosphate buffer, pH 6.0, at 4 °C and concentrated in a Vivaspin (10,000 Da cutoff for *Lm*ChiA and 30,000-Da cutoff for other enzymes). The protein concentration of the sample was determined by the Bradford method using a commercial kit (Bio-Rad) with bovine γ-globulin as a protein reference standard. Alternatively, protein concentration was measured with Qubit 2.0 fluorometer (Invitrogen) according to the manufacturer's protocol. Commercially available enzymes of *S. griseus* (Sigma) and *T. viride* (a mixture of two chitinases; Sigma) were dissolved according to manufacturer's instructions and included in the study as reference enzymes.

For the enzymes from *Salmonella*, *Sodalis*, *Polysphondylium*, and *Listeria*, purified in this study, 12–73 mg of recombinant protein were obtained per liter of cell culture after chromatography on HisTrap columns. The purity of proteins after isolation assessed by SDS-PAGE was as found previously (14).

Glycan Array Scanning—Glycan arrays were fabricated as described previously (22, 23) and contained 317 carbohydrate ligands (supplemental Table 1). The chips were first treated for 30 min with blocking buffer (50 mM ethanolamide in 50 mM borate buffer, pH 8.0) and subsequently for 30 min with 0.1 M phosphate-buffered saline (PBS) (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 138 mM NaCl, and 2.7 mM KCl, pH 7.4) (Sigma) containing 0.1% Tween 20 (ICN, MP Biomedicals, Santa Ana, CA). 1 ml of 200 μg/ml enzyme (*St*ChiA, *St*ChiA E223Q, and *St*ChiA E223QΔCBM) diluted in PLI-P buffer (0.5 M NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 1% BSA, 1% Triton-X-100, pH 7.4), pH 7.4, was added to the slide and incubated for 60 min at 37 °C. The slide was washed in 1×PBS (0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, 0.138 M NaCl, and 0.0027 M KCl, pH 7.4) (Sigma) containing 0.1% Tween 20 (ICN), then 1 ml of rabbit anti-ChiA polyclonal antibody (CovalAb UK Ltd, Cambridge, UK) diluted to 1/1000 in PLI-P buffer was added, and

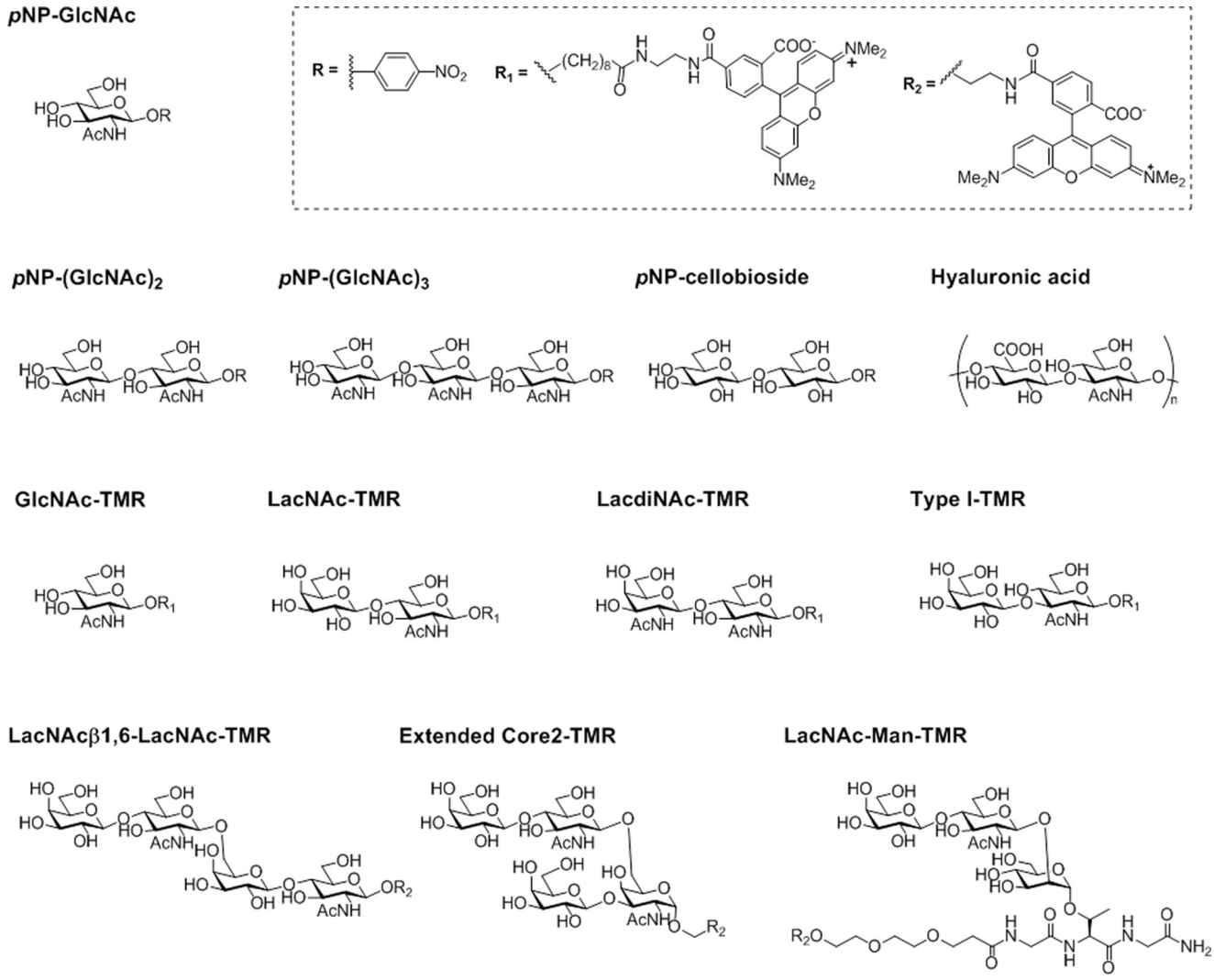


FIGURE 1. **Overview of substrates used in this study.**

the slide was incubated for 60 min at 22 °C. The slide was washed with 1×PBS, then 1 ml of labeled secondary antibody (Cy5 goat anti-rabbit IgG (H+L); Invitrogen) diluted 1/500 in PLI-P buffer was added before incubation for 60 min at 22 °C. The slide was then washed with 1×PBS, spin-dried, and stored in the dark until analysis. Fluorescence intensities were detected using a ScanArray 5000 (PerkinElmer Life Sciences) confocal scanner, and image analyses were carried out by using ScanArray Express 3.0 and the fixed 70-μm-diameter rings method as well as Microsoft EXCEL software.

Examination of Enzyme Activity—A survey of the activity of chitinases was initially carried out with the *p*NP chitin pseudo-substrates: *p*NP-GlcNAc, *p*NP-$(GlcNAc)_2$, *p*NP-$(GlcNAc)_3$, and the cellulose pseudo-substrate 4-nitrophenyl-β-D-cellobioside whose structures are shown in Fig. 1. In the survey, 5 μl of enzyme (40–2000 μg/ml) diluted in 50 mM sodium phosphate buffer, pH 6.0, and 45 μl of 1.8 mM substrate dissolved in 50 mM sodium phosphate buffer, pH 6.0, were incubated at 30 °C. To limit the conversion of substrates to a maximum of 15–20%, the following enzyme concentrations were used: *Sg*ChiA (400 μg/ml), *St*ChiAΔCBM (200 μg/ml), *Pp*Chitinase (100 μg/ml), *Lm*ChiA (40 μg/ml), *Lm*ChiB (200 μg/ml), *Lm*ChiBΔCBM (200 μg/ml), *T. viride* mixture (67 μg/ml) and *S. griseus* (400 μg/ml). 50-μl samples were removed after 30 min, and the reaction was quenched by adding 250 μl of 0.4 M sodium carbonate (Sigma S2127). Absorbance at 405 nm was measured in a plate reader and corrected for absorption using a control sample with added sodium phosphate buffer, pH 6.0, instead of enzyme. After the initial screening, *p*NP-$(GlcNAc)_2$ absorption values were converted into concentrations by the use of a *p*-nitrophenol (Sigma) standard curve. Conversion rates were normalized against protein concentration and are listed as pmol/min/mg of protein.

Kinetic parameters for *p*NP-$(GlcNAc)_2$ were determined for *Sg*ChiA (23 μg/ml) and *Pp*Chitinase (20 μg/ml) by measuring initial rates of reaction at eight different substrate concentrations ranging from 0.007 to 1.8 mM during 25–120 min of incubation at 30 °C. K_m and V_{max} were calculated using the software GraphPad Prism 4.0 (GraphPad Software), and k_{cat} was estimated by dividing V_{max} by enzyme concentration.

The activity of chitinases toward hyaluronic acid (Sigma, H5388) was tested in a standard enzymatic assay of hyaluronidase (Sigma) using hyaluronidase (bovine testes, Sigma H3506)

as a positive control. Samples were prepared according to the manufacturer's instructions and incubated at 37 °C for up to 40 h, and absorbance was read as % transmittance (%T) at 600 nm.

Fluorescently labeled model substrates had a TMR tag covalently linked to carbohydrates via a hydrophobic linker ((carbohydrate)β-O-$(CH_2)_8$CONH$(CH_2)_2$NHCO-TMR) (24, 25). The structures are shown in Fig. 1. Initial screening was carried out by TLC analysis where 1 μl of GlcNAc-TMR (1 mM), LacNAc-TMR (20 mM), LacdiNAc-TMR (20 mM), or Type I-TMR (Galβ1–3GlcNAc-TMR, 1 mM) were incubated with 5 μl of enzyme at ambient temperature for 2–24 h. Reaction progress was monitored by removing 1-μl aliquots for thin-layer chromatography on silica gel plates developed with $CHCl_3$/MeOH/H_2O (65/35/5) as described previously (17). The TMR-labeled compounds, which are brightly colored red/purple, can be visualized by eye. The *T. viride* chitinase mixture was used as a positive control for monitoring GlcNAc-TMR and LacdiNAc-TMR hydrolysis.

After screening by TLC, conversion rates of LacNAc-TMR and LacdiNAc-TMR for all chitinases were determined by monitoring product formation with 5 mM substrate at 30 °C by capillary electrophoresis. The reaction volume was 4 μl in 50 mM sodium phosphate buffer, pH 6.0, and enzyme concentrations were 0.6–2.5 mg/ml. Aliquots (1 μl) were removed at 120 min and quenched with 50 μl of CE running buffer (50 mM borate, pH 9.3, and 150 mM sodium dodecyl sulfate). Analyses were performed on an automated PrinCE 560 CE system from PrinCE Technologies B.V. (Emmen, The Netherlands). Conversion rates were normalized against protein concentration and are listed as pmol/min/mg of protein. The enzyme concentrations for assay with LacdiNAc/LacNAc were: *Sg*ChiA (2000 μg/ml), *St*ChiAΔCBM (67/400 μg/ml), *Pp*Chitinase (2000 μg/ml), *Lm*ChiA (2000 μg/ml), *Lm*ChiB (200/2000 μg/ml), *Lm*ChiBΔCBM (200/2000 μg/ml), *T. viride* mixture (20/200 μg/ml), *S. griseus* (1000 μg/ml). An independent study of conversion rates of LacNAc-TMR, LacdiNAc-TMR, and *p*NP-$(GlcNAc)_2$ for all chitinases was performed using the same substrate concentrations as above but with fixed enzyme concentrations of 200 μg/ml.

Kinetic parameters for LacNAc-TMR and LacdiNAc-TMR were determined for *Sodalis*, *Salmonella*, and *Polysphondylium* enzymes by monitoring product formation with 8 different concentrations of substrate ranging from 0.080 to 10.5 mM at 30 °C by capillary electrophoresis. The reaction volume was 10 μl in 50 mM sodium phosphate buffer, pH 6.0, and enzyme concentrations were 0.6–2.5 mg/ml. Aliquots (1 μl) were removed at 12–120 min and quenched with 50 μl of CE running buffer as described above. Analyses were performed as described above. Separations were carried out in an uncoated fused-silica capillary of 75-μm inner diameter with CE running buffer. TMR-labeled compounds were detected and quantitated using an Argos 250B fluorescence detector (Flux Instruments, Basel, Switzerland) equipped with an excitation filter of 546.1/10 nm and an emission filter of 570 nm. All experiments were carried out at a normal polarity, *i.e.* inlet anodic. Data were processed with the PrinCE 7.0 software.

Ultra-HPLC-mass spectrometry analysis was employed to screen extended substrates terminating in LacNAc including LacNAcβ1–6LacNAcβ-TMR, LacNAcβ1–6(Galβ1–3)GalNAcα-TMR (extended core 2), and LacNAcβ1–2-Man-TMR substrates (Fig. 1), which are only available in limited amounts (26, 27). Assays were carried out in 4 μl containing 50 mM sodium phosphate buffer, pH 6.0, 1 μg of each enzyme, and 5 pmol of TMR-substrate. After overnight incubation at room temperature, a 1-μl aliquot was removed, mixed with 300 μl of buffer (10 mM ammonium formate, pH 4.5 (22%), acetonitrile (88%) (v/v)) for analysis by ultra-HPLC-MS as previously described (17).

Nuclear Magnetic Resonance—Substrates, either *p*NP-$(GlcNAc)_2$, $(GlcNAc)_3$, LacNAc-TMR, or LacdiNAc-TMR (0.4–1 mg), were dissolved in 0.7 ml of buffer, transferred to 5-ml nuclear magnetic resonance (NMR) tubes, and standard one-dimensional ^{1}H NMR spectra of substrates were acquired before and after the addition of 5–50 μl of undiluted *Sg*ChiA and *St*ChiAΔCBM (LacdiNAc-TMR only). NMR spectra were recorded at 15 or 25 °C on a Bruker Avance 800 instrument and analyzed as previously described (14). Deuterated buffer was prepared by lyophilizing 5 ml of 50 mM sodium phosphate buffer, pH 6.0, followed by the addition of 2 ml of ^2H_2O to the dried solids, re-lyophilization, and suspension in 5 ml of ^2H_2O. Spectra were recorded at 799.3 MHz using tetramethylsilane ($\delta = 0$ ppm) as the internal standard with a 32 scan composite presaturation.

RESULTS

Phylogenetic Analyses—A phylogenetic analysis of selected family 18 glycosyl hydrolase catalytic domains revealed that they cluster roughly into three distinct groups (colored in *red*, *green*, and *blue*, respectively, in Fig. 2). The groups colored in *green* and *blue* fit into Cluster A according to the analysis of Karlsson and Stenlid (28). Catalytic domains in these two groups in general carry a chitin-insertion domain conferring a deep substrate binding cleft, which suggests exo-activity (29). The group colored in blue contains *St*ChiA, *Sg*ChiA, and *Pp*Chitinase from *Salmonella*, *Sodalis*, and *Polysphondylium*, respectively, and is characterized by a lack of signal peptides, yet all are predicted to be secretory proteins. On the other hand, the green group containing *Lm*ChiB from *Listeria* and also human chitinases in general carry signal peptides. The group colored in *red* (Fig. 2) containing *Lm*ChiA fit into group IV in cluster B according to Karlsson and Stenlid (28). The red group is characterized by the lack of a chitin-insertion domain, which confers a shallow substrate binding cleft suggesting endo-activity (29).

Glycan Array Scanning—The glycan array scanning was performed to screen for ligand binding by the full-length active *Salmonella St*ChiA enzyme as well as inactive variants with or without the chitin binding domain. Average background signal was below 1000 relative fluorescence units (RFU), and average signals above 3000 RFU were considered as positives. The glycan array scanning showed binding of the inactive *Salmonella* chitinases (*St*ChiA E223Q and *St*ChiA E223QΔCBM) to GlcNAc-containing glycans including the chitin oligosaccha-

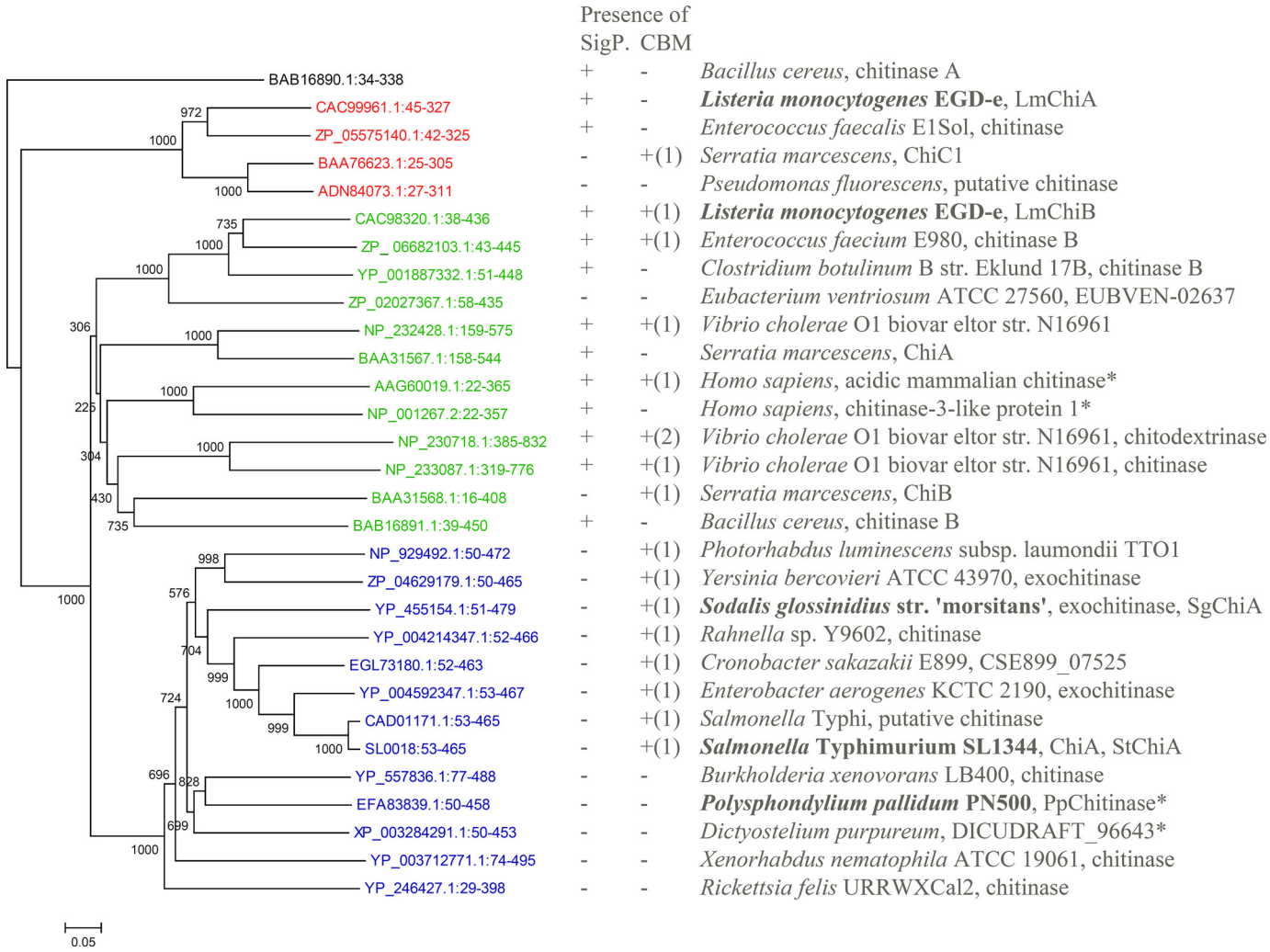


FIGURE 2. **Phylogenetic tree of GH_18 domains including *Salmonella* Typhimurium ChiA, *L. monocytogenes* ChiA and ChiB, *S. glossinidius* ChiA, and *P. pallidum* chitinase (all in bold) with representative sequences for similar domains in other species and strains.** Also shown is the presence of N-terminal signal peptide (*SigP*) and chitin binding modules (*CBM*). *Bar*, 0.05 substitutions per nucleotide position. Tree labels are colored to indicate phylogenetic grouping. Descriptions of enzymes are from the sequence annotations. Eukaryotic sequences (*Homo sapiens*, *P. pallidum*, and *Dictyostelium purpureum*) are marked with an *asterisk* (*).

TABLE 2

Affinity of *Salmonella* *St*ChiA and the inactive variants *St*ChiA E223Q and *St*ChiA E223QΔCBM for glycans immobilized on a glass slide (glycan array screening)

−, average signal is below 3000 relative fluorescence units (RFU); +, average signal is >3000 RFU; ++, average signal is >10,000 RFU; +++, average signal is >15,000 RFU. TF, Thomsen-Friedenreich Galβ1–3GalNAc; LN, *N*-acetyllactosamine (LacNAc); sp2, -O$(CH_2)_2NH_2$, sp3: -O$(CH_2)_3NH_2$; sp4, -NHCOCH_2NH_2.

Glycan no.	Glycan name	*St*ChiA	*St*ChiA E223Q	*St*ChiA E223Q ΔCBM
493	Chitopentaose (GlcNAcβ1–4)$_5$β-sp4	−	+++	+++
503	Chitohexaose (GlcNAcβ1–4)$_6$-sp4	−	+++	+++
384	LNβ3′LN-Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ-sp2	+++	+++	++
385	LNβ3′LN Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ-sp3	+++	++	+++
420	(4′SuLN)β3′LN 4-O-SuGalβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ-sp3	++	+	+
498	(LN)$_3$ (Galβ1–4GlcNAcβ1–3)$_3$-sp3	+++	+	+
229	Ab3′LN Galβ1–3Galβ1–4GlcNAcβ-sp4	+	−	−
88	TF$_\beta$-antigen Galβ1–3GalNAcβ-sp3	+	−	−
89	TF-antigen Galβ1–3GalNAcα-sp3	+	−	−
110	Maltose Glcα1–4Glcβ-sp3	+	−	−
390	Maltotetraose (Glcα1–4)$_4$β-sp4	++	−	−

rides chitopentaose and chitohexaose (glycan no. 493 and 503; Table 2, supplemental Table 1). The active enzyme (*St*ChiA) did not bind to the latter two compounds, unlike the inactive variants, showing that hydrolytic activity interfered with glycan binding. The active enzyme also bound to a range of non-chitinous substrates with binding to five LacNAc-containing compounds (compounds No. 384, 385, 420, 498, 229; Table 2, supplemental Table 1) as well as TF (Thomsen-Friedenreich antigen Galβ1–3GalNAc) antigen and TF$_\beta$ antigen structures (glycan number 88 and 89, respectively; Table 2 and supple-

TABLE 3

Overview of the initial substrate activity screening of *Salmonella*, *Sodalis*, *Polysphondylium* and *Listeria* chitinases monitored by spectrophotometric *p*NP-assay or TLC

For the *p*NP assay: +, positive reaction ($ABS_{405} > 0.1$); (+), weak positive reaction ($ABS_{405} = 0.07–0.1$); −, negative reaction ($ABS_{405} < 0.06$). For the TLC assay: +, clear red/purple band visible to the eye; (+), weak red/purple band but visible to the eye; −, no band visible to the eye. For the hyaluronidase activity assay: %T, % transmittance at 600 nm.

Assay	Substrate[a]	*St*ChiAΔCBM	*Sg*ChiA	*Pp*Chitinase	*Lm*ChiA	*Lm*ChiB	*Lm*ChiBΔCBM	*T. viride* mixture	*S. griseus* chitinase
*p*NP assay	*p*NP-GlcNAc	−	−	−	−[b]	NT	NT	+	−
	*p*NP-$(GlcNAc)_2$	+	+	+	+	+	+	+	+
	*p*NP-$(GlcNAc)_3$	+	(+)	NT	+[b]	NT	NT	+	(+)
	*p*NP-cellobioside	−	−	−	−[b]	NT	NT	−	−
TLC assay	GlcNAc-TMR	−	−	−	−	−	NT	+	−
	LacNAc-TMR	+	+	−	−	(+)	(+)	−	−
	LacdiNAc-TMR	+	+	+	+	+	+	+	−
	Type I-TMR	−	−	−	−	−	NT	−	−
%T	Hyaluronic acid	−	−	−	−	−	−	(+)	−

[a] *p*NP-GlcNAc: 4-nitrophenyl-*N*-acetyl- β-D-glucosaminide; *p*NP-$(GlcNAc)_2$, 4-nitrophenyl-*N,N′*-diacetyl-β-D-chitobioside; *p*NP-$(GlcNAc)_3$, 4-nitrophenyl-β-D-*N,N′,N″*-triacetylchitotriose; *p*NP-cellobioside, 4-nitrophenyl-β-D-cellobioside; NT, not tested.
[b] Not tested in this study. Data are from Leisner *et al.* (21).

mental Table 1). The inactive enzymes only bound to some of these substrates (compound numbers 384, 385, 420, and 498) suggesting that changes in the active site also affected binding.

Substrate Specificity and Kinetic Parameters—The activity spectrum of chitinases was initially screened by the use of the chitin pseudo-substrates *p*NP-GlcNAc, *p*NP-$(GlcNAc)_2$, *p*NP-$(GlcNAc)_3$, and *p*NP-cellobioside, the mammalian and insect model substrates LacNAc-TMR and LacdiNAc-TMR, Type I-TMR (Galβ1–3GlcNAc-TMR isomer of LacNAc-TMR), and hyaluronic acid (Table 3). All of the tested chitinases demonstrated activity toward the chitin analogs *p*NP-$(GlcNAc)_2$ and *p*NP-$(GlcNAc)_3$. None of the chitinases tested was active against *p*NP-cellobioside, and only the reference enzyme *T. viride* chitinase mixture demonstrated activity toward *p*NP-GlcNAc and hyaluronic acid. All chitinases except the *S. griseus* chitinase were to varying degrees active toward the substrate LacdiNAc-TMR. The *St*ChiAΔCBM and *Sg*ChiA and to some extent also the *Lm*ChiB chitinase were active against LacNAc-TMR. The latter results were confirmed by use of capillary electrophoresis except for *Lm*ChiB (Tables 4 and 5). None of the chitinases were active against the isomeric Type I-TMR (Table 3).

Conversion rates in pmol/min/mg of protein were determined for all chitinases toward *p*NP-$(GlcNAc)_2$, LacNAc-TMR, and LacdiNAc-TMR, and clear differences among the chitinases were observed. For *p*NP-$(GlcNAc)_2$, the *Lm*ChiA was the fastest converter (after the reference *T. viride* chitinase) followed by *St*ChiAΔCBM. The *Salmonella* enzyme *St*ChiAΔCBM was the fastest converter of LacdiNAc-TMR (Table 4).

We further studied the kinetics of the three phylogenetically related enzymes. Both *St*ChiAΔCBM and *Sg*ChiA hydrolyzed LacNAc-TMR and LacdiNAc-TMR with greater activity toward LacdiNAc-TMR for both enzymes as seen by the higher k_{cat} value (Table 5, Fig. 3). *St*ChiAΔCBM had a k_{cat} for LacdiNAc-TMR of 0.18 s^{-1}, which is 25% that of the k_{cat} for the chitinase substrate *p*NP-$(GlcNAc)_2$, whereas k_{cat}/K_m was 20% that of *p*NP-$(GlcNAc)_2$. The k_{cat} for LacNAc-TMR hydrolysis was 0.046 s^{-1} about 7% of *p*NP-$(GlcNAc)_2$. For *Sg*ChiA, the k_{cat} value for LacdiNAc-TMR was 0.035 s^{-1} compared with a k_{cat} value of 0.015 s^{-1} for LacNAc-TMR hydrolysis. The *Pp*Chitinase also showed some activity against LacdiNAc-TMR, although it was slower than the *St*ChiAΔCBM and *Sg*ChiA as seen from the low k_{cat} value of 0.007 s^{-1}. On the other hand, the *Pp*Chitinase was more active against *p*NP-$(GlcNAc)_2$ than *St*ChiAΔCBM and *Sg*ChiA as seen from the high k_{cat} value of 10.3 s^{-1} compared with 0.7 and 3.1 s^{-1} for *St*ChiAΔCBM and *Sg*ChiA, respectively (Table 5).

TABLE 4

Conversion rates for hydrolysis of LacdiNAc-TMR, LacNAc-TMR, and *p*NP-$(GlcNAc)_2$ by *Salmonella*, *Sodalis*, *Polysphondylium*, and *Listeria* chitinases

Enzyme	Conversion rates of substrate		
	LacdiNAc-TMR[a]	LacNAc-TMR[a]	*p*NP - $(GlcNAc)_2$[b]
	pmol/min/mg enzyme		
*Sg*ChiA	8.3 ± 0.0	5.1 ± 2.6	320 ± 60
*St*ChiAΔCBM	151 ± 3	28.0 ± 0.1	2460 ± 40
*Pp*Chitinase	5.0 ± 0.3	0.0 ± 0.0	1570 ± 350
*Lm*ChiA	5.0 ± 0.1	0.6 ± 0.6	5660 ± 520
*Lm*ChiB	33 ± 13	0.1 ± 0.2	1750 ± 50
*Lm*ChiBΔCBM	47.1 ± 0.0	0.0 ± 0.1	1630 ± 80
T. viride chitinase mixture	1232 ± 50	0.2 ± 0.3	6700 ± 80
S. griseus chitinase	0.9 ± 0.7	0.3 ± 0.3	1380 ± 40

[a] 5 mM substrate, 30 °C, 120 min, analysis by capillary electrophoresis.
[b] 1.8 mM substrate, 30 °C, 30 min analysis by spectrophotometry.

Hydrolysis of larger substrates with terminal LacNAc (Fig. 1) was monitored by ultra-HPLC-MS due to the limited amounts of material available. Only LacNAcβ1–6LacNAcβ-TMR was a substrate. It was hydrolyzed by stepwise removal of terminal LacNAc forming LacNAc-TMR disaccharide. Subsequent hydrolysis of LacNAc-TMR can yield the TMR linker (Fig. 4*B*). The LacNAc disaccharides that are cleaved are not detected by the fluorescence detector used in our analysis. The *Sodalis* *Sg*ChiA enzyme was the most efficient with respect to hydrolysis with essentially complete conversion to a mixture of LacNAc-TMR (65%) and the TMR-linker (33%). *Lm*ChiB was the least efficient enzyme with conversion to 3% each of LacNAc-TMR and the TMR-linker. *Lm*ChiA showed no activity with this extended substrate, whereas the *St*ChiAΔCBM enzyme only removed the terminal LacNAc giving 62% LacNAc-TMR (Fig. 4, *A* and *C*).

Nuclear Magnetic Resonance Studies of Sodalis and Salmonella Chitinases with pNP-(GlcNAc)$_2$, pNP-(GlcNAc)$_3$, LacNAc-TMR, and LacdiNAc-TMR—LacdiNAc-TMR and LacNAc-TMR were shown to be substrates of *Sg*ChiA and *St*ChiAΔCBM with release of LacdiNAc and LacNAc, respectively, as moni-

TABLE 5
Kinetic constants for *Salmonella*, *Sodalis*, and *Polysphondylium* chitinases with *p*NP-$(GlcNAc)_2$, LacNAc-TMR, and LacdiNAc-TMR

Enzyme	*p*NP-$(GlcNAc)_2$			LacNAc-TMR[a]			LacdiNAc-TMR[z]		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
	mM	s^{-1}	$mM^{-1}s^{-1}$	mM	s^{-1}	$mM^{-1}s^{-1}$	mM	s^{-1}	$mM^{-1}s^{-1}$
*St*ChiAΔCBM	0.73[b]	0.7[b]	0.95	2.8 ± 0.1	0.046	0.017	1.0 ± 0.2	0.18	0.18
*Sg*ChiA	0.29 ± 0.09	3.1	10.7	4.7 ± 0.4	0.015	0.003	0.39 ± 0.16	0.0355	0.09
*Pp*Chitinase	1.1 ± 0.4	10.3	9.6				2.9 ± 0.4	0.007	0.002

[a] Monitored by capillary electrophoresis.
[b] Not tested in this study. Data are from Larsen *et al.* (14).

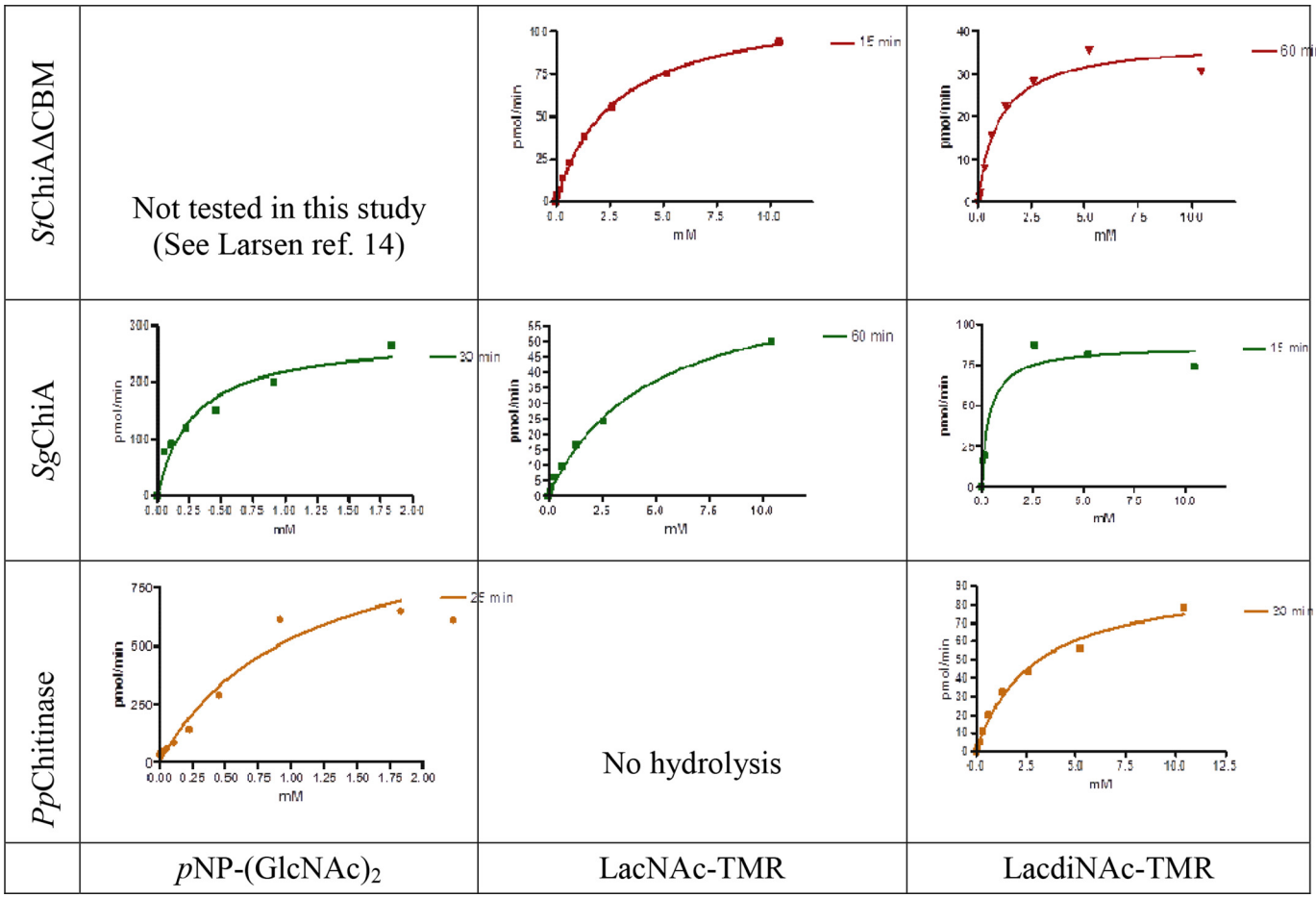


FIGURE 3. **Kinetics of hydrolysis by *Salmonella*, *Sodalis*, and *Polysphondylium* chitinases of *p*NP-$(GlcNAc)_2$, LacNAc-TMR, and LacdiNAc-TMR at 30 °C.**

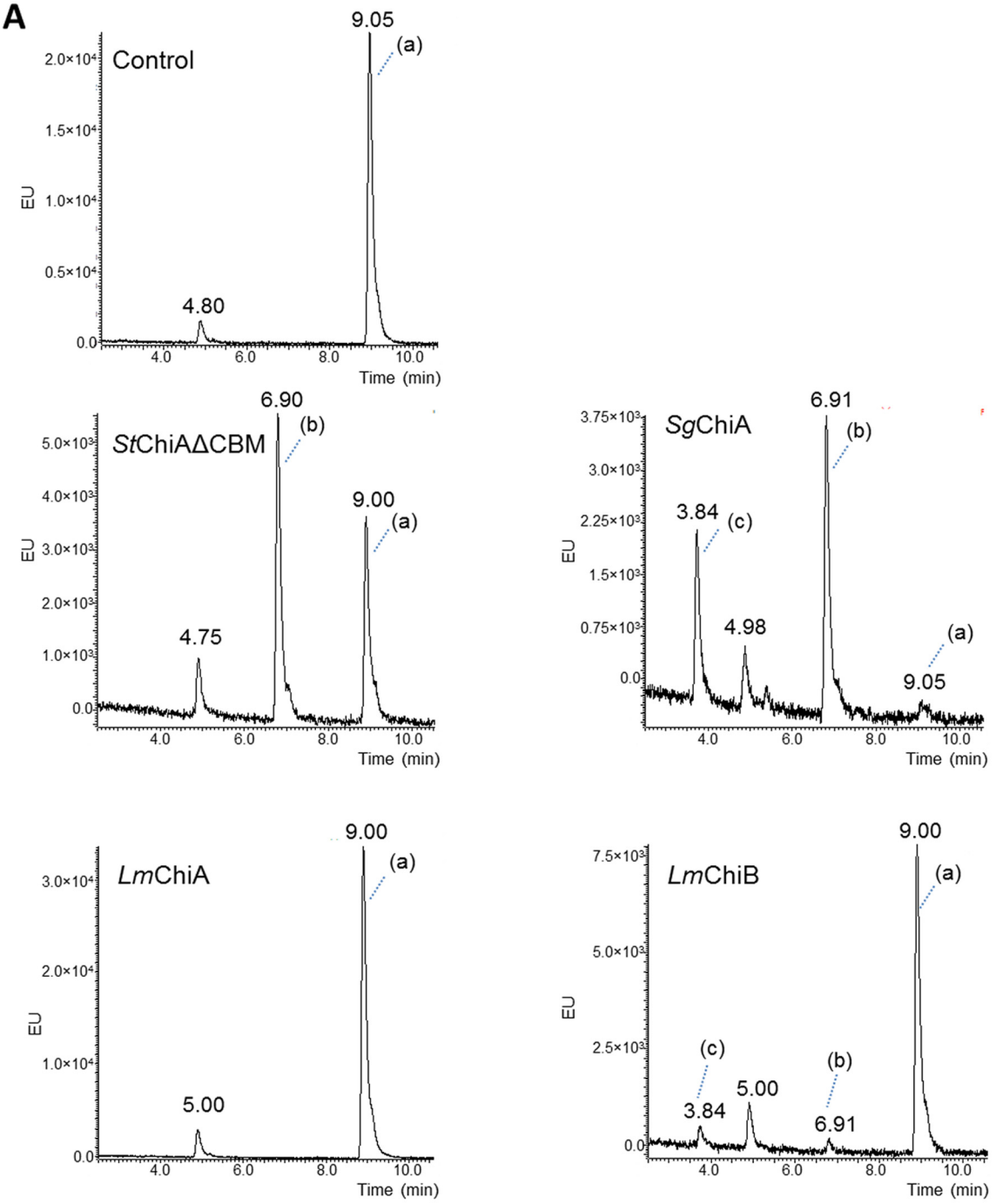
tored by ^{1}H NMR. LacdiNAc consists of a GalNAc and a GlcNAc unit, and these two units gave distinct anomeric signals in ^{1}H NMR as can be seen in Fig. 5, which shows the hydrolysis of LacdiNAc-TMR by *Sg*ChiA. From the bottom, the first spectrum (Fig. 5*A*) shows the substrate before adding enzyme with the peak at 4.18 ppm from H1 of GlcNAc and the peak at 4.33 ppm from H1 of GalNAc. The spectra B, C, and D show the formation of the product, LacdiNAc, at 1, 3.5, and 9 h, respectively, after the addition of the enzyme. The peak at 4.37 ppm is the signal from H1 of β-GalNAc, when the reducing end is β-GlcNAc, and the peak at 4.38 ppm is the signal from H1 of β-GalNAc when the reducing end is α-GlcNAc. The signal at 4.55 ppm is the H1 of β-GlcNAc reducing end, and the signal at 5.19 ppm is the H1 of α-GlcNAc reducing end. Note that the α-anomer is formed later than the reducing end β-anomer thus confirming that the reaction occurs with retention of configuration as the reducing end β-anomer is the only product of enzyme-catalyzed steps.

*p*NP-chitobiose, *p*NP-$(GlcNAc)_2$, and chitotriose $(GlcNAc)_3$ were also shown to be substrates of *Sg*ChiA as monitored by NMR with release of β-disaccharide $(GlcNAc)_2$ from *p*NP-$(GlcNAc)_2$ and release of β-monosaccharide and β-disaccharide from the trisaccharide $(GlcNAc)_3$, and slow mutarotation to α-mono- and α-disaccharide (data not shown). These are analogous to the results previously reported for *Salmonella* *St*ChiAΔCBM (14).

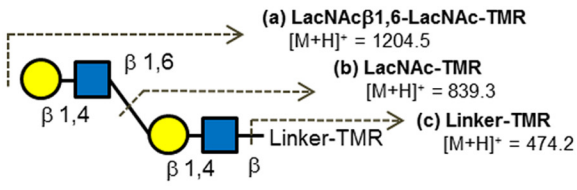
DISCUSSION

We have investigated the affinity of a *Salmonella* chitinase toward a large range of substrates by use of a glycan microarray technique followed by investigations of conversion rates of

A



B



C

Enzyme	Composition of TMR Compounds (%)			Hydrolytic Conversion (%)
	(a)	(b)	(c)	
*St*ChiAΔCBM	38	62	0	62
*Sg*ChiA	2	65	33	98
*Lm*ChiA	100	0	0	n.d.
*Lm*ChiB	94	3	3	6

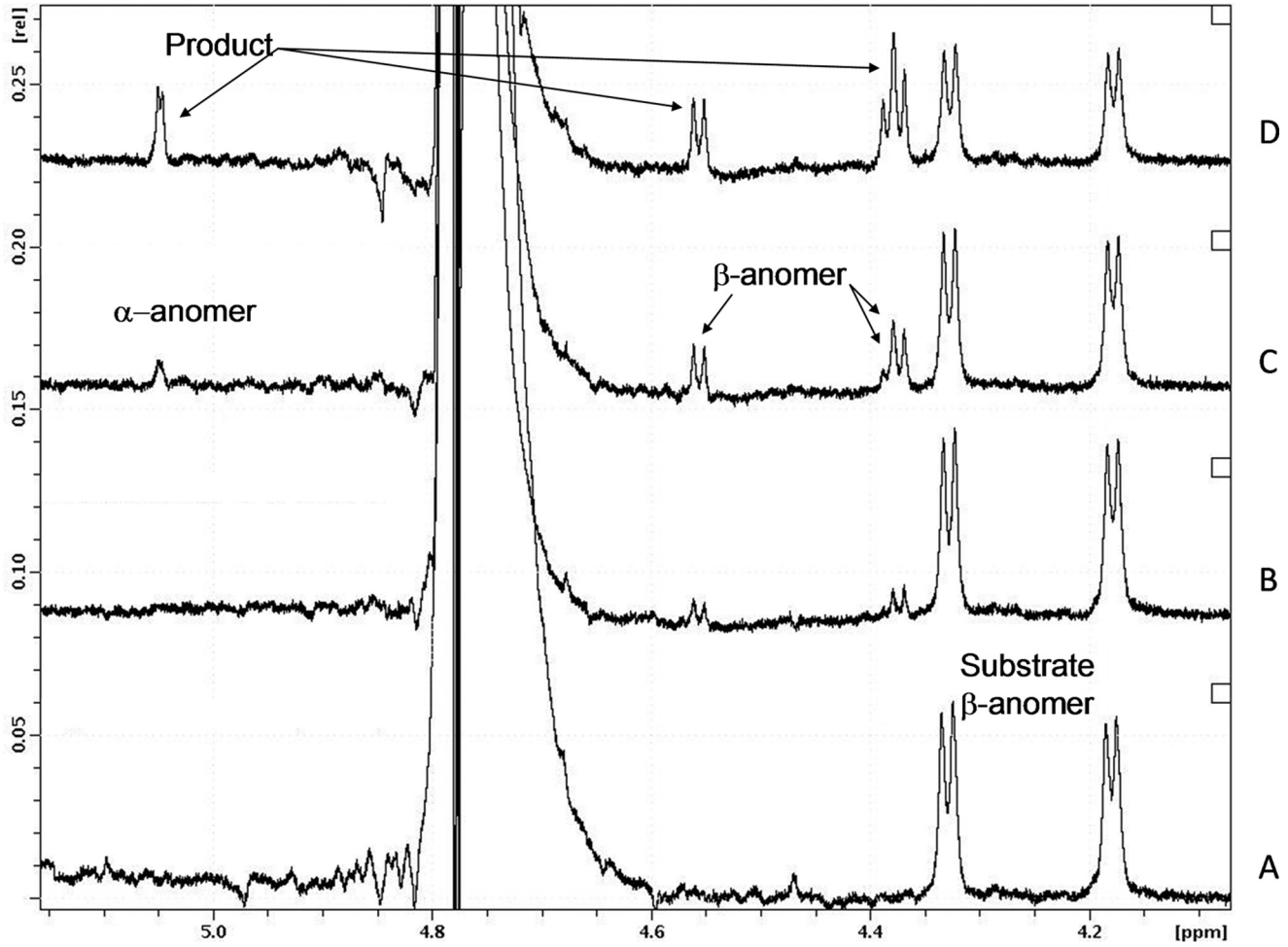


FIGURE 5. **Hydrolysis of LacdiNAc-TMR by *Sodalis* chitinase (*Sg*ChiA) monitored by one-dimensional proton NMR showing only the anomeric region.** *A*, substrate before the addition of enzyme. The signal at 4.18 ppm is H1 of GlcNAc and 4.33 ppm H1 of GalNAc. *B–D* show LacdiNAc formed at 1 h (*B*), 3.5 h (*C*), and 9h (*D*) after the addition of enzyme. The signal at 4.37 ppm is H1 of β-GalNAc, when the reducing end is β-GlcNAc. The signal at 4.38 ppm is H1 of β-GalNAc when the reducing end is α. The signal at 4.55 ppm is β-GlcNAc reducing end, and 5.19 ppm is α-GlcNAc reducing end.

selected substrates by several chitinases and, finally, by screening a small selection of potential biological substrates.

The glycan array scanning highlighted a specific affinity of the full-length *Salmonella* chitinase, *St*ChiA, toward various GlcNAc-containing glycans. As expected, chitin-like substrates such as chitopentaose and chitohexaose were targeted by this chitinase. Some GlcNAc-containing glycans of mammalian-like LacNAc structures were also targets (Table 2). LacNAc structures have been identified as terminating glycans of glycoproteins and glycolipids on vertebrate cells and in the human glycome (30, 31). The inclusion of the inactive *Salmonella* variants *St*ChiA E223Q and *St*ChiA E223QΔCBM in the study ensured that a reliable overview of binding affinities was obtained.

Detailed analyses of hydrolysis of the most promising of these mammalian and/or insect-like substrates were examined by use of a wider range of bacterial and eukaryotic chitinases. The chitinases were selected from a broad phylogenetic range and included the related (Fig. 2) enzymes from the Enterobacteriaceae *Salmonella* Typhimurium and *S. glossinidius* as well as the slime mold *P. pallidum* and the unrelated chitinases from the Firmicute *L. monocytogenes*. Some of the enzymes (the *Listeria Lm*ChiB and *Sodalis Sg*ChiA) encode additional domains annotated as chitin binding domains, whereas others contain only the catalytic domain (*Lm*ChiA, *St*ChiAΔCBM, *Polysphondylium Pp*Chitinase; Fig. 2). Additional studies are needed to clearly distinguish the respective roles of the family 18 glycosyl hydrolase domain and additional domains. The observation of marginal differences in hydrolytic activities for *Lm*ChiB and *Lm*ChiBΔCBM without the chitin binding domain (Tables 3 and 4) suggest that there is a minimal effect of the chitin binding domain on the hydrolytic activities under the conditions tested.

The enzymatic analyses were performed using the fluorescently labeled model substrates LacdiNAc-TMR and LacNAc-TMR. A general activity toward LacdiNAc-TMR was observed

FIGURE 4. **Ultra-HPLC-mass spectrometry analyses of LacNAcβ1,6-LacNAc-TMR before and after reaction with four different chitinases.** *A*, chromatograms of the reaction mixtures were monitored with a fluorescence detector ((λ_{ex} = 540 nm; λ_{em} = 580 nm), and each peak was identified by mass spectrometry. *EU*, emission units. *B*, structures of the substrate (*a*) and the products (LacNAc-TMR (*b*) and Linker-TMR (*c*)), corresponding to labels on the chromatograms. The *yellow circle* and the *blue square* indicate Gal and GlcNAc, respectively. *C*, hydrolytic reaction yields based on peak areas of the TMR products formed. For a presentation of the exact structures, please see Fig. 1 and supplemental Fig. 1.

for all of the chitinases examined, except *S. griseus* chitinase, with the release of LacdiNAc from LacdiNAc-TMR. This is consistent with LacdiNAc binding to the −2 and −1 subsites and the hydrophobic linker with TMR in the enzymes + subsites. The hydrolytic activity toward LacNAc-TMR was much lower, and only the *Sg*ChiA and *St*ChiAΔCBM released LacNAc from LacNAc-TMR to any extent within the 2-h incubation time (Table 4). The increased activity toward LacdiNAc-TMR is attributed to the presence of two *N*-acetyl groups, on the reducing end for substrate-assisted catalysis and on the non-reducing end for additional recognition by the respective chitinases. It is notable that the *T. viride* enzyme mixture also exhibited a high degree of hydrolytic activity toward LacdiNAc-TMR. Further analyses are needed to elucidate which *T. viride* chitinase(s) is responsible for this activity and to evaluate the potential biological role. The general activity of chitinases toward a LacdiNAc-terminating substrate is a novel observation only reported previously for the human chitotriosidase (17).

Hydrolysis of LacNAc model substrates is also not commonly reported. In addition to the previous studies of a *Salmonella* chitinase and the human chitotrisidase by the authors of the present paper (14, 17) only two studies have demonstrated such activity expressed by the chitinases in the bacterial species *Amycolatopsis orientalis* (32) and *Bacillus circulans* (33). The *Amycolatopsis* chitinase not only hydrolyzed chitobiose from *p*NP-$(GlcNAc)_2$ but also released free oligosaccharides and *p*-nitrophenol from Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ-*p*NP GlcNAcβ1,3Galβ1,4GlcNAcβ-*p*NP, and LacNAc-*p*NP (Galβ1,4GlcNAcβ-*p*NP). LacNAc and LacNAc-*p*NP were not detected in the course of tetrasaccharide hydrolysis, consistent with *p*NP occupying the +1 subsite and the tetrasaccharide occupying the enzymes −4 to −1 subsites.

To demonstrate that the activity toward LacdiNAc-TMR and LacNAc-TMR was not a minor side activity of the chitinases, we examined the substrate specificities and Michaelis-Menten kinetic parameters for LacdiNAc-TMR, LacNAc-TMR, and *p*NP-$(GlcNAc)_2$ for *St*ChiAΔCBM, *Sg*ChiA, and *Pp*Chitinase.

*St*ChiAΔCBM and *Sg*ChiA showed similar trends in hydrolytic activity toward LacNAc-TMR and LacdiNAc-TMR, with an increased activity for LacdiNAc-TMR for both enzymes as seen by the 2–4 times higher k_{cat} values (Table 5). When comparing k_{cat} and k_{cat}/K_m values for LacNAc- and LacdiNAc-TMR to the higher k_{cat} and k_{cat}/K_m values for the chitin pseudo-substrate *p*NP-$(GlcNAc)_2$, the k_{cat} value for the *St*ChiAΔCBM enzyme with LacdiNAc-TMR was still 25% that of the value observed with *p*NP-$(GlcNAc)_2$ (Table 5). Even higher k_{cat} values toward LacdiNAc-TMR relative to pNP-$(GlcNAc)_2$ have been observed for the human chitotriosidase (17). The rather low level of activity indicated by the low k_{cat} values observed for the *Pp*Chitinase toward LacdiNAc-TMR (Table 5) should be interpreted with caution.

For *Sg*ChiA and *St*ChiAΔCBM the LacdiNAc motif may be a potential biological target as this substrate is commonly found in the insect glycome (34). *Salmonella* has been associated with cockroaches, *Chironomus* midges and flies (35–38), and *S. glossinidius* is an endosymbiont of the tsetse fly (*Glossina* spp., Ref. 39).

The presence of chitinases in *P. pallidum* may provide an activity of general importance, such as degradation of yeast prey, as they are also found in another major cellular slime mold genus, *Dictyostelium* (Fig. 2); these two genera mainly belong to different groups within the Dictyostelia (40, 41).

To investigate whether the observed activities toward LacNAc-TMR and LacdiNAc-TMR are biologically relevant, we screened the *Listeria*, *Salmonella*, and *Sodalis* chitinases (*Lm*ChiA and *Lm*ChiB, *St*ChiAΔCBM, and *Sg*ChiA, respectively) for activities toward extended substrates including LacNAcβ1–6LacNAcβ-TMR, LacNAcβ1–6(Galβ1–3)GalNAcα-TMR (extended core 2), and LacNAcβ1–2-Man-TMR (Fig. 1). Only LacNAcβ1–6LacNAcβ-TMR was a substrate. It is of interest to note that the two enzymes (*St*ChiAΔCBM and *Sg*ChiA) that showed a high catalytic activity toward this substrate both were associated with the phylogenetic group colored in *blue* and belonging to cluster A (Fig. 2; Ref. 28). Further studies are, however, necessary to demonstrate that this group of chitinases indeed has an increased preference for this target.

The stepwise hydrolysis pattern for LacNAcβ1–6LacNAcβ-TMR is consistent with the terminal LacNAc occupying the −2 and −1 subsites and the inner LacNAc-TMR occupying the +1 and +2 subsites. The hydrolysis of LacNAcβ1–6LacNAcβ-TMR can be attributed to the flexibility of the β1,6 linkage. The product of hydrolysis is LacNAcβ-TMR, which in turn was further hydrolyzed by *Sg*ChiA and *Lm*ChiB enzymes. In this case the linker occupies the + subsites. The product distribution profiles indicate that the tetrasaccharide is hydrolyzed twice as rapidly as LacNAc-TMR by the *Sg*ChiA, whereas they are equally good substrates for *Lm*ChiB. The inability of *St*ChiAΔCBM to further hydrolyze LacNAcβ-TMR might be due to the shorter linker to TMR in this compound ($(CH_2)_2$) compared with the linker to LacNAc-TMR used for kinetics ($(CH_2)_8$) or to a greater affinity for tetrasaccharide such that it precludes binding and hydrolysis of LacNAcβ-TMR. This highlights caution in the interpretation of kinetic results when non-natural leaving groups such as fluorescent linkers or the widely used *p*NP are used. The hydrolysis patterns were distinct from those seen for *Amycolatopsis* chitinase, which bound all oligosaccharide substrates with *p*NP in the +1 subsite. These results are in agreement with what has recently been observed for the human chitotriosidase (17). It can be concluded that terminal LacNAc or LacdiNAc structure-linked β1,4 or β1,6 in oligosaccharides, glycoproteins, or glycolipids are potential substrates for several chitinases. Especially for the *Salmonella* *St*ChiAΔCBM and *Sodalis* *Sg*ChiA, this activity was significant, whereas this was not for the case for the *Listeria* chitinases (Fig. 4). Interestingly, it has been proposed that GH_18 enzymes from the human gut bacterial symbiont *Bacteroides thetaiotaomicron* may target LacNAc repeats of *O*-glycan chains (42).

It can be suggested that for *Salmonella* *St*ChiA and *Sodalis* *Sg*ChiA chitinases, glycans that contain either a LacNAc or LacdiNAc motif constitute part of targets that contribute to a biological response, most likely in association with infection of mammalian hosts (*Salmonella*) or symbiosis with insects (*Sodalis*). This hypothesis is in agreement with the observation that the gene expression of the *Salmonella* chitinase is markedly up-regulated during infection of macrophage and epithe-

lial cells (15, 16). We are currently conducting studies aimed at identifying the potential biological substrates for a selection of these enzymes including the *Listeria* and *Salmonella* chitinases.

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REFERENCES

1. Keyhani, N. O., and Roseman, S. (1999) Physiological aspects of chitin catabolism in marine bacteria. *Biochim. Biophys. Acta* **1473,** 108–122
2. Kuranda, M. J., and Robbins, P. W. (1991) Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **266,** 19758–19767
3. Merzendorfer, H., and Zimoch, L. (2003) Chitin metabolism in insects: structure, function, and regulation of chitin synthases and chitinases. *J. Exp. Biol.* **206,** 4393–4412
4. Schlumbaum, A., Mauch, F., Vögeli, U., and Boller, T. (1986) Plant chitinases are potent inhibitors of fungal growth. *Nature* **324,** 365–367
5. Taira, T. (2010) Structures and antifungal activity of plant chitinases. *J. Appl. Glycosci.* **57,** 167–176
6. Kasprzewska, A. (2003) Plant chitinases: regulation and function. *Cell. Mol. Biol. Lett.* **8,** 809–824
7. Shih, N. R., McDonald, K. A., Jackman, A. P., Girbés, T., and Iglesias, R. (1997) Bifunctional plant defence enzymes with chitinase and ribosome inactivating activities from *Trichosanthes kirilowii* cell cultures. *Plant Sci.* **130,** 145–150
8. Lee, C. G., Da Silva, C. A., Dela Cruz, C. S., Ahangari, F., Ma, B., Kang, M.-J., He, C.-H., Takyar, S., and Elias, J. A. (2011) Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Annu. Rev. Physiol.* **73,** 479–501
9. Lee, C. G., Herzog, E. L., Ahangari, F., Zhou, Y., Gulati, M., Lee, C.-M., Peng, X., Feghali-Bostwick, C., Jimenez, S. A., Varga, J., and Elias, J. A. (2012) Chitinase 1 is a biomarker for and therapeutic target in scleroderma-associated interstital lung disease that augments TGF-β1 signaling. *J. Immunol.* **189,** 2635–2644
10. Boot, R. G., van Achterberg, T. A., van Aken, B. E., Renkema, G. H., Jacobs, M. J. H. M., Aerts, J. M. F. G., and de Vries, C. J. M. (1999) Strong induction of members of the chitinase family of proteins in atherosclerosis. Chitotriosidase and human cartilage gb-39 expressed in lesion macrophages. *Arterioscler. Thromb. Vasc. Biol.* **19,** 687–694
11. Di Rosa, M., Dell'Ombra, N., Zambito, A. M., Malaguarnera, M., Nicoletti, F., and Malaguarnera, L. (2006) Chitotriosidase and inflammatory mediator levels in Alzheimer's disease and cerebrovascular dementia. *Eur. J. Neurosci.* **23,** 2648–2656
12. Chaudhuri, S., Gantner, B. N., Ye, R. D., Cianciotto, N. P., and Freitag, N. E. (2013) The *Listeria monocytogenes* ChiA chitinase enhances virulence through suppression of host innate immunity. *MBio* **4,** e00617–e00612
13. Frederiksen, R. F., Paspaliari, D. K., Larsen, T., Storgaard, B. G., Larsen, M. H., Ingmer, H., Palcic, M. M., and Leisner, J. J. (2013) Bacterial chitinases and chitin-binding proteins as virulence factors. *Microbiology* **159,** 833–847
14. Larsen, T., Petersen, B. O., Storgaard, B. G., Duus, J. Ø., Palcic, M. M., and Leisner, J. J. (2011) Characterization of a novel *Salmonella* Typhimurium chitinase which hydrolyzes chitin, chitooligosaccharides and an *N*-acetyllactosamine conjugate. *Glycobiology* **21,** 426–436
15. Eriksson, S., Lucchini, S., Thompson, A., Rhen, M., and Hinton, J. C. D. (2003) Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol. Microbiol.* **47,** 103–118
16. Hautefort, I., Thompson, A., Eriksson-Ygberg, S., Parker, M. L., Lucchini, S., Danino, V., Bongaerts, R. J. M., Ahmad, N., Rhen, M., and Hinton, J. C. (2008) During infection of epithelial cells *Salmonella enterica* serovar Typhimurium undergoes a time-dependent transcriptional adaptation that results in simultaneous expression of three type 3 secretion systems. *Cell. Microbiol.* **10,** 958–984
17. Larsen, T., Yoshimura, Y., Voldborg, B. G. R., Cazzamali, G., Bovin, N. V., Westerlind, U., Palcic, M. M., and Leisner, J. J. (2014) Human chitotriosidase CHIT1 cross reacts with mammalian-like substrates. *FEBS Lett.* **588,** 746–751
18. Leisner, J. J., Larsen, M. H., Jørgensen, R. L., Brøndsted, L., Thomsen, L. E., and Ingmer, H. (2008) Chitin hydrolysis by *Listeria* spp., including *L. monocytogenes*. *Appl. Environ. Microbiol.* **74,** 3823–3830
19. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Res.* **25,** 4876–4882
20. Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24,** 1596–1599
21. Leisner, J. J., Larsen, M. H., Ingmer, H., Petersen, B. O., Duus, J. Ø., and Palcic, M. M. (2009) Cloning and comparison of phylogenetically related chitinases from *Listeria monocytogenes* EGD and *Enterococcus faecalis* V583. *J. Appl. Microbiol.* **107,** 2080–2087
22. Blixt, O., Head, S., Mondala, T., Scanlan, C., Huflejt, M. E., Alvarez, R., Bryan, M. C., Fazio, F., Calarese, D., Stevens, J., Razi, N., Stevens, D. J., Skehel, J. J., van Die, I., Burton, D. R., Wilson, I. A., Cummings, R., Bovin, N., Wong, C.-H., and Paulson, J. C. (2004) Printed covalent glycan array for ligand profiling of diverse glycan binding proteins. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 17033–17038
23. Shilova, N., Navakouski, M., Khasbiullina, N., Blixt, O., and Bovin, N. (2012) Printed glycan array: antibodies as probed in undiluted serum and effects of dilution. *Glycoconj. J.* **29,** 87–91
24. Zhang, Y., Le, X., Dovichi, N. J., Compston, C. A., Palcic, M. M., Diedrich, P., and Hindsgaul, O. (1995) Monitoring biosynthetic transformations of *N*-acetyllactosamine using fluorescently labeled oligosaccharides and capillary electrophoretic separation. *Anal. Biochem.* **227,** 368–376
25. Le, X. C., Zhang, Y., Dovichi, N. J., Compston, C. A., Palcic, M. M., Beever, R. J., and Hindsgaul, O. (1997) Study of the enzymatic transformation of fluorescently labeled oligosaccharides in human epidermoid cells using capillary electrophoresis with laser-induced fluorescence detection. *J. Chromatogr. A* **781,** 515–522
26. Goy, R., Apfel, U.-P., Elleouet, C., Escudero, D., Elstner, M., Göerls, H., Talarmin, J., Schollhammer, P., González, L., and Weigand, W. (2013) A silicon-heteroaromatic system as photosensitizer for light-driven hydrogen production by hydrogenase mimics. *Eur. J. Inorg. Chem.* **2013,** 4466–4472
27. Severov, V. V., Belyanchikov, I. M., Pazynina, G. V., and Bovin, N. V. (2007) Synthesis of *N*-acetyl-lactosamine-containing oligosaccharides, galectin ligands. *Russ. J. Bioorg. Chem.* **33,** 122–138
28. Karlsson, M., and Stenlid, J. (2009) Evolution of family 18 glycoside hydrolases: diversity, domain structures, and phylogenetic relationships. *J. Mol. Microbiol. Biotechnol.* **16,** 208–223
29. Horn, S. J., Sørbotten, A., Synstad, B., Sikorski, P., Sørlie, M., Vårum, K. M., and Eijsink, V. G. H. (2006) Endo/exo mechanism and processivity of family 18 chitinases produced by *Serratia marcescens*. *FEBS J.* **273,** 491–503
30. Cummings, R. D. (2009) The repertoire of glycan determinants in the human glycome. *Mol. Biosyst.* **5,** 1087–1104
31. Yu, T., Guo, C., Wang, J., Hao, P., Sui, S., Chen, X., Zhang, R., Wang, P., Yu, G., Zhang, L., Dai, Y., and Li, N. (2011) Comprehensive characterization of the site-specific *N*-glycosylation of wild-type and recombinant human lactoferrin expressed in the milk of transgenic cloned cattle. *Glycobiology* **21,** 206–224
32. Murata, T., Amarume, S., Hattori, T., Tokuyama, S., Tokuyasu, K., Kawagishi, H., and Usui, T. (2005) Purification and characterization of a chitinase from *Amycolatopsis orientalis* with N-acetyllactosamine-repeating unit releasing activity. *Biochem. Biophys. Res. Commun.* **336,** 514–520

33. Shoda, S.-I., Misawa, Y., Nishijima, Y., Tawata, Y., Kotake, T., Noguchi, M., Kobayashi, A., and Watanabe, T. (2006) Chemo-enzymatic synthesis of novel oligo-*N*-acetyllactosamine derivatives having a $\beta(1-4)$-$\beta(1-6)$ repeating unit by using transition state analogue substrate. *Cellulose* **13,** 477–484
34. North, S. J., Koles, K., Hembd, C., Morris, H. R., Dell, A., Panin, V. M., and Haslam, S. M. (2006) Glycomic studies of *Drosophila melanogaster* embryos. *Glycoconj. J.* **23,** 345–354
35. Kopinac, R. J., Sheldon, B. W., and Wright, C. G. (1994) Cockroaches as vectors of *Salmonella:* laboratory and field trials. *J. Food Prot.* **57,** 125–132
36. Olsen, A. R., and Hammack, T. S. (2000) Isolation of *Salmonella* spp. from the housefly, *Musca domestica* L., and the dump fly, *Hydrotaea aenescens* (Wiedemann) (Diptera: Muscidae), at caged-layer houses. *J. Food Prot.* **63,** 958–960
37. Moore, B. C., Martinez, E., Gay, J. M., and Rice, D. H. (2003) Survival of *Salmonella enterica* in freshwater and sediments and transmission by the aquatic midge *Chironomus tentans* (Chironomidae: Diptera). *Appl. Environ. Microbiol.* **69,** 4556–4560
38. Holt, P. S., Geden, C. J., Moore, R. W., and Gast, R. K. (2007) Isolation of *Salmonella enterica* serovar Enteritidis from houseflies (*Musca domestica*) found in rooms containing *Salmonella* serovar Enteritidis-challenged hens. *Appl. Environ. Microbiol.* **73,** 6030–6035
39. Dale, C., Young, S. A., Haydon, D. T., and Welburn, S. C. (2001) The insect endosymbiont *Sodalis glossinidius* utilizes a type III secretion system for cell invasion. *Proc. Natl. Acad. Sci. U.S.A.* **98,** 1883–1888
40. Schaap, P., Winckler, T., Nelson, M., Alvarez-Curto, E., Elgie, B., Hagiwara, H., Cavender, J., Milano-Curto, A., Rozen, D. E., Dingermann, T., Mutzel, R., and Baldauf, S. L. (2006) Molecular phylogeny and evolution of morphology in the social amoebas. *Science* **314,** 661–663
41. Bonner, J. T. (2009) *The Social Amoebae: the Biology of Cellular Slime Molds*, pp. 22–23, Princeton University Press, Princeton, NJ
42. Martens, E. C., Chiang, H. C., and Gordon, J. I. (2008) Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. *Cell Host Microbe* **4,** 447–457

A Diverse Range of Bacterial and Eukaryotic Chitinases Hydrolyze the LacNAc (Galβ1-4GlcNAc) and LacdiNAc (GalNAcβ1-4GlcNAc) Motifs Found on Vertebrate and Insect Cells.

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Supplementary Table 1. Layout of glycan structures of glycan array.

# in the print&plate layouts	Nr.	Spacered form of saccharide	Common name	Short name	Molecular weight	Molecular weight of OS with TFA	V, ul of 10 mM solution
		Monosaccharides					
1	**001**	**Fucα-sp3**	L-α-Fuc	aF	221.3		2
2	**002**	**Galα-sp3**	α-Gal	aA	237.3		2
3	**003**	**Galβ-sp3**	β-Gal	bA	237.3	351.3	2
4	**004**	**GalNAcα-sp0**	TnSer	TnSer	308.3		2
5	**005**	**GalNAcα-sp3**	T_n	Tn	278.3	392.3	2
6	**006**	**GalNAcβ-sp3**	β-GalNAc	bAN	278.3	392.3	2
7	**007**	**Glcα-sp3**	α-Glc	aG	237.3	351.3	2
8	**009**	**Glcβ-sp3**	β-Glc	bG	237.3		2
9	**010**	**GlcNAcβ-sp3**	β-GlcNAc	GN	278.3	392.3	2
10	**011**	**GlcNAcβ-sp2**	β-GlcNAc	GN-C2	264.3	378.3	2
11	**012**	**GlcNAcβ-sp7**	β-GlcNAc	GN-Ph	312.3		2
12	**013**	**GlcNAcβ-sp8**	β-GlcNAc	GN-PEG	484.6	598.6	2
13	**014**	**GlcN(Gc)β-sp4**	β-GlcN(Gc)	bGN(Gc)	293.3		2
14	**015**	**$HOCH_2(HOCH)_4CH_2NH_2$**	aminoglucitol	glucitol	181.2		2
15	**016**	**Manα-sp3**	α-Man	aM	237.3	351.3	2
16	**017**	**Manα-sp4**	α-Man	aM-Gly	236.2		2
17	**018**	**Manβ-sp4**	β-Man	bM	236.2	350.2	2
18	**019**	**ManNAcβ-sp4**	β-ManNAc	bMN	277.3	391.3	2

19	**020**	**Rhaα-sp3**	L-α-Rha	aR	221.3	335.3	2
291	**021**	**GalNAcα-sp4**	β-GalNAc	bAN-Gly	277.3		2
292	**022**	**GlcNAcβ-sp4**	β-GlcNAc	GN-Gly	277.3		2
20	**037**	**3-O-Su-Galβ-sp3**	3-O-Su-β-Gal	bA3Su	317.3		2
21	**040**	**4-O-Su-GalNAcβ-sp4**	4-O-Su-β-GalNAc	bAN4Su-Gly	357.3		2
22	**043**	**6-O-Su-GlcNAcβ-sp3**	6-O-Su-β-GlcNAc	GN6Su	358.4		2
23	**044**	**GlcAα-sp3**	α-glucuronic acid	aGU	251.2		2
24	**045**	**GlcAβ-sp3**	β-glucuronic acid	bGU	251.2		2
25	**046**	**6-H_2PO_3Glcβ-sp4**	β-Glc6P	G6P	316.2		2
26	**047**	**6-H_2PO_3Manα-sp3**	α-Man6P	M6P	339.2 (Na^+)		2
27	**048**	**Neu5Acα-sp3**	α-Neu5Ac	Sia	366.4		2
28	**049**	**Neu5Acα-sp9**	α-Neu5AcBn	Sia-Bn	471.5		2
29	**050**	**Neu5Acβ-sp3**	β-Neu5Ac	bSia	366.4		2
30	**051**	**No formula available**					2
31	**052**	**Neu5Gcα-sp3**	α-Neu5Gc	aNeu5Gc	382.4		2
32	**053**	**Neu5Gcβ-sp3**	β-Neu5Gc	bSia5Gc	382.4		2
33	**054**	**9-NAc-Neu5Acα-sp3**	9-NAc-α-Neu5Ac	9NAcSia	407.4		2
290	**055**	**3-O-Su-GlcNAcβ-sp3**	3-O-Su-β-GlcNAc-	GN3Su	358.4		2
		Disaccharides					
34	**071**	**Fucα1-2Galβ-sp3**	H_{di}	Hdi	383.4	497.4	2
35	**072**	**Fucα1-3GlcNAcβ-sp3**		Fa3GN	424.5	538.5	2
36	**073**	**Fucα1-4GlcNAcβ-sp3**	Le	Le	424.5		2
37	**074**	**No formula available**					2
38	**075**	**Galα1-2Galβ-sp3**		Aa2A	399.4		2
39	**076**	**Galα1-3Galβ-sp3**	B_{di}	Bdi	399.4	513.4	2
40	**077**	**Galα1-3GalNAcβ-sp3**	$T_{\alpha\beta}$	Tab	440.5		2
41	**078**	**Galα1-3GalNAcα-sp3**	$T_{\alpha\alpha}$	Taa	440.5	554.5	2

42	**079**	**No formula available**					2
43	**080**	**Galα1-3GlcNAcβ-sp3**		Aa3GN	440.5		2
44	**081**	**Galα1-4GlcNAcβ-sp3**	α-LacNAc	aLN	440.5	554.5	2
45	**082**	**Galα1-4GlcNAcβ-sp8**	α-LacNAc	aLN-PEG	646.7	760.7	2
46	**083**	**Galα1-6Glcβ-sp4**	melibiose	Aa6G	398.4	512.4	2
47	**084**	**Galβ1-2Galβ-sp3**		Ab2A	399.4	513.4	2
48	**086**	**Galβ1-3GlcNAcβ-sp2**	Le^c	LeC-C2	426.4		2
49	**087**	**Galβ1-3Galβ-sp3**		Ab3A	399.4	513.4	2
50	**088**	**Galβ1-3GalNAcβ-sp3**	$T_{\beta\beta}$	Tbb	440.5		2
51	**089**	**Galβ1-3GalNAcα-sp3**	TF	TF	440.5	554.5	2
52	**090**	**No formula available**					2
53	**092**	**Galβ1-4Glcβ-sp2**	Lac	Lac-C2	385.4	499.4	2
54	**093**	**Galβ1-4Glcβ-sp4**	Lac	Lac-Gly	398.4		2
55	**094**	**Galβ1-4Galβ-sp4**		Ab4A	398.4	512.4	2
56	**095**	**Galβ1-4GlcNAcβ-OCH_2CH_2NH(Et)**	LacNAc	LN-C2Et	454.5		2
57	**097**	**Galβ1-4GlcNAcβ-sp3**	LacNAc	LN	440.5	554.5	2
58	**098**	**Galβ1-4GlcNAcβ–sp5**	LacNAc	LN-C8	553.6		2
59	**099**	**Galβ1-4GlcNAcβ-sp8**	LacNAc	LN-PEG	646.7	760.7	2
60	**100**	**Galβ1-6Galβ-sp4**		Ab6A	398.4	512.4	2
61	**101**	**GalNAcα1-3GalNAcβ-sp3**	Fs-2	Fs2	481.5	595.5	2
62	**102**	**GalNAcα1-3Galβ-sp3**	A_{di}	Adi	440.5	554.5	2
63	**103**	**GalNAcα1-3GalNAcα-sp3**	core 5	core5	481.5		2
64	**104**	**GalNAcβ1-3Galβ-sp3**		ANb3A	440.5	554.5	2
65	**106**	**GalNAcβ1-4GlcNAcβ-sp3**	LacdiNAc	LacdiNAc	481.5		2
66	**107**	**GalNAcβ1-4GlcNAcβ-sp2**	LacdiNAc	LacdiNAc-C2	467.5	581.5	2
67	**109**	**$GalNAc_{(furanose)}$β1-4GlcNAcβ-sp2**		ANfb4GN-C2	467.5		2
68	**110**	**Glcα1-4Glcβ-sp3**	maltose	Malt2	399.4		2
69	**111**	**Glcβ1-4Glcβ-sp4**	cellobiose	cello	398.4	512.4	2
70	**112**	**Glcβ1-6Glcβ-sp4**	gentiobiose	gent	398.4		2

71	**113**	**GlcNAcβ1-3GalNAcα-sp3**	core 3	core3	481.5		2
72	**114**	**GlcNAcβ1-3Manβ-sp4**		GN3M	439.4	553.4	2
73	**115**	**GlcNAcβ1-4GlcNAcβ-Asn**	chitobiose-Asn	Ch2-Asn	538.5		2
74	**117**	**GlcNAcβ1-4GlcNAcβ-sp4**	chitobiose	Ch2-Gly	480.5	594.5	2
75	**118**	**GlcNAcβ1-6GalNAcα-sp3**	core 6	core6	481.5	595.5	2
76	**119**	**Manα1-2Manβ-sp4**		Ma2Mb	398.4	512.4	2
77	**120**	**Manα1-3Manβ-sp4**		Ma3M	398.4	512.4	2
78	**121**	**Manα1-4Manβ-sp4**		Ma4M	398.4	512.4	2
79	**122**	**Manα1-6Manβ-sp4**		Ma6M	398.4		2
80	**123**	**No formula available**					2
81	**124**	**Manα1-2Manα-sp4**		Ma2Ma	398.4	512.4	2
293	**125**	**(6-Bn-Galβ1-4)GlcNAcβ-sp2**	6'Bn-LacNAc	6'Bn-LN	516.6		2
294	**126**	**6-Bn-Galα1-4(6-Bn)GlcNAcβ-sp**	Bn_2-α-LacNAc	Bn2-aLN	606.7		2
295	**127**	**Galβ1-4Glcβ-sp4-Phe**	Lac	Lac-Phe	545.6		2
296	**128**	**Galβ1-4Glcβ-sp4-Trp**	Lac	Lac-Trp	584.6		2
82	**143**	**Fucα1-2(3-O-Su)Galβ-sp3**	3-O-Su-H_{di}	Hdi3Su	463.5		2
83	**144**	**Galβ1-3(6-O-Su)GlcNAcβ-sp2**	6-O-Su-Le^c	LeC6Su-C2	506.5		2
84	**145**	**Galβ1-3(6-O-Su)GlcNAcβ-sp3**	6-O-Su-Le^c	LeC6Su	520.5		2
85	**146**	**Galβ1-4(6-O-Su)Glcβ-sp2**	6-O-Su-Lac	Lac6Su	465.4		2
86	**147**	**Galβ1-4(6-O-Su)GlcNAcβ-sp3**	6-O-Su-LacNAc	LN6Su	520.5		2
87	**148**	**GalNAcβ1-4(6-O-Su)GlcNAcβ-sp2**	6-O-Su-LacdiNAc	LacdiNAc6Su	547.5		2
88	**149**	**GlcNAcβ1-4(6-O-Su)GlcNAcβ-sp2**	6-O-Su-chitobiose	Ch2-6Su	547.5		2
89	**150**	**3-O-Su-Galβ1-3GalNAcα-sp3**	3'-O-Su-TF	TF3'Su	520.5		2
90	**151**	**6-O-Su-Galβ1-3GalNAcα-sp3**	6'-O-Su-TF	TF6'Su	520.5		2
91	**152**	**3-O-Su-Galβ1-4Glcβ-sp2**	SM3	Lac3'Su	465.3		2
92	**153**	**6-O-Su-Galβ1-4Glcβ-sp2**	6'-O-Su-Lac	Lac6'Su	465.3		2
93	**154**	**3-O-Su-Galβ1-3GlcNAcβ-sp2**	3'-O-Su-Le^c	LeC3'Su-C2	506.5		2
94	**155**	**3-O-Su-Galβ1-3GlcNAcβ-sp3**	3'-O-Su-Le^c	LeC3'Su	520.5		2

95	**156**	**3-O-Su-Galβ1-4GlcNAcβ-sp2**	3'-O-Su-LacNAc	LN3'Su-C2	506.5		2
96	**157**	**3-O-Su-Galβ1-4GlcNAcβ-sp3**	3'-O-Su-LacNAc	LN3'Su	520.5		2
97	**158**	**4-O-Su-Galβ1-4GlcNAcβ-sp2**	4'-O-Su-LacNAc	LN4'Su-C2	506.5		2
98	**159**	**4-O-Su-Galβ1-4GlcNAcβ-sp3**	4'-O-Su-LacNAc	LN4'Su	520.5		2
99	**160**	**6-O-Su-Galβ1-3GlcNAcβ-sp2**	6'-O-Su-Le^c	LeC6'Su-C2	506.5		2
100	**161**	**6-O-Su-Galβ1-3GlcNAcβ-sp3**	6'-O-Su-Le^c	LeC6'Su	520.5		2
101	**162**	**6-O-Su-Galβ1-4GlcNAcβ-sp2**	6'-O-Su-LacNAc	LN6'Su-C2	506.5		2
102	**164**	**GlcAβ1-3GlcNAcβ-sp3**		GUb3GN	454.3		2
103	**165**	**GlcAβ1-3Galβ-sp3**		GUb3A	413.4		2
297	**166**	**GlcAβ1-6Galβ-sp3**		GUb6A	413.4		2
104	**167**	**GlcNAcβ1-4-[HOOC(CH_3)CH]-3-O-GlcNAcβ-sp4**	GlcNAc-Mur	GN-Mur	552.5		2
105	**168**	**GMDP-Lys**	GMDP-Lys	GMDPLys	823.9		2
106	**169**	**Neu5Acα2-3Galβ-sp3**	GM4	GM4	528.5		2
107	**170**	**Neu5Acα2-6Galβ-sp3**		Sia6A	528.5		2
108	**171**	**Neu5Acα2-3GalNAcα-sp3**	3-SiaT_n	3-SiaTn	569.6		2
109	**172**	**Neu5Acα2-6GalNAcα-sp3**	SiaT_n	SiaTn	569.6		2
110	**173**	**Neu5Acβ2-6GalNAcα-sp3**	β-SiaT_n	bSiaTn	569.6		2
111	**174**	**Neu5Gcα2-6GalNAcα-sp3**	Neu5Gc-T_n	Neu5GcTn	569.6		2
112	**175**	**Neu5Gcβ2-6GalNAcα-sp3**	βNeu5Gc-T_n	bNeu5GcTn	585.6		2
113	**176**	**3-O-Su-Galβ1-4(6-O-Su)Glcβ-sp2**	3',6-di-O-Su-Lac	Lac3',6Su2	567.5 (Na^+)		2
114	**177**	**3-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp3**	3',6-di-O-Su-LacNAc	LN3'6Su2	622.6 (Na^+)		2
115	**179**	**6-O-Su-Galβ1-3(6-O-Su)GlcNAcβ-sp2**	6,6'-di-O-Su-Le^c	LeC6,6'Su2	608.5 (Na^+)		2
116	**180**	**6-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp2**	6,6'-di-O-Su-LacNAc	LN66'Su2	608.5 (Na^+)		2
117	**181**	**No formula available**					2
118	**182**	**3,6-O-Su_2-Galβ1-4GlcNAcβ-sp2**	3',6'-di-O-Su-LacNAc	LN3'6'Su2	608.5 (Na^+)		2
119	**183**	**4,6-O-Su_2-Galβ1-4GlcNAcβ-sp2**	4',6'-di-O-Su-LacNAc	LN4'6'Su2-C2	608.5 (Na^+)		2
120	**184**	**4,6-O-Su_2-Galβ1-4GlcNAcβ-sp3**	4',6'-di-O-Su-	LN4'6'Su2	622.6 (Na^+)		2

			LacNAc				
121	**186**	**Neu5Acα2-8Neu5Acα2-sp3**	$(Sia)_2$	(Sia)2	679.6 (Na^+)		2
122	**187**	**No formula available**					2
123	**188**	**No formula available**					2
298	**189**	**3,6-O-Su_2-Galβ1-4(6-O-Su)GlcNAcβ-sp2**	3',6,6'-tri-O-Su-LacNAc	LN3'66'Su3	710.6 ($2Na^+$)		2
299	**190**	**Galβ1-4-(6-P)GlcNAcβ-sp2**	6P-LacNAc	LN6P	506.4		2
300	**191**	**6-P-Galβ1-4GlcNAcβ-sp2**	6'P-LacNAc	LN6'P	506.4		2
301	**192**	**GalNAcβ1-4(6-O-Su)GlcNAcβ-sp3**	6-O-Su-LacdiNAc	LacdiNAc6Su	561.5		2
302	**193**	**3-O-Su-GalNAcβ1-4GlcNAcβ-sp3**	3'-O-Su-LacdiNAc	LacdiNAc3'Su	561.5		2
303	**194**	**6-O-Su-GalNAcβ1-4GlcNAcβ-sp3**	6'-O-Su-LacdiNAc	LacdiNAc6'Su	561.5		2
304	**195**	**6-O-Su-GalNAcβ1-4-(3-O-Ac)GlcNAcβ-sp3**	6'-Su-3-O-Ac-LacdiNAc	3Ac-LacdiNAc6'Su	603.5		2
305	**196**	**3-O-Su-GalNAcβ1-4(3-O-Su)-GlcNAcβ-sp3**	3,3'-O-Su_2-LacdiNAc	LacdiNAc3,3'Su2	663.5(Na^+)		2
306	**197**	**3,6-O-Su_2-GalNAcβ1-4-GlcNAcβ-sp3**	3',6'-Su_2-LacdiNAc	LacdiNAc3',6'Su2	663.5(Na^+)		2
307	**198**	**4,6-O-Su_2-GalNAcβ1-4GlcNAcβ-sp3**	4',6'-O-Su_2-LacdiNAc	LacdiNAc4',6'Su2	663.5(Na^+)		2
308	**199**	**4,6-O-Su_2-GalNAcβ1-4-(3-O-Ac)GlcNAcβ-sp3**	4',6'-Su_2-3-O-Ac-LacdiNAc	3Ac-LacdiNAc4',6'Su2	705.5(Na^+)		2
309	**200**	**4-O-Su-GalNAcβ1-4GlcNAcβ-sp3**	4'-O-Su-LacdiNAc	LacdiNAc4'Su	561.5		2
310	**201**	**3,4-O-Su_2-GalNAcβ1-4-GlcNAcβ-sp3**	3',4'-Su_2-LacdiNAc	LacdiNAc3',4'Su2	663.5(Na^+)		2
		Trisaccharides					
124	**215**	**Fucα1-2Galβ1-3GlcNAcβ-sp3**	Le^d, H (type 1)	LeD	586.6	700.6	2
125	**216**	**Fucα1-2Galβ1-4GlcNAcβ-sp3**	H (type 2)	Htype2	586.6	700.6	2
126	**217**	**Fucα1-2Galβ1-3GalNAcα-sp3**	H (type 3)	Htype3	586.6	700.6	2
127	**219**	**Fucα1-2Galβ1-4Glcβ-sp4**	H (type 6)	Htype6	544.5	658.5	2

128	**220**	**Galα1-3Galβ1-4Glcβ-sp2**		Aa3'Lac-C2	547.5		2
129	**221**	**Galα1-3Galβ1-4Glcβ-sp4**		Aa3'Lac-Gly	560.5	674.5	2
130	**222**	**Galα1-3Galβ1-4GlcNAcβ-sp3**	Galili (tri)	Galili3	602.6		2
131	**223**	**Galα1-4Galβ1-4Glcβ-sp2**	P^k, Gb3, $GbOse_3$	Pk-C2	547.5		2
132	**224**	**Galα1-4Galβ1-4Glcβ-sp3**	P^k, Gb3, $GbOse_3$	Pk	561.5		2
133	**225**	**Galα1-4Galβ1-4GlcNAcβ-sp2**	P_1	P1	588.6		2
134	**226**	**Galα1-3 Galβ-sp3 Fucα1-2**	B_{tri}	Btri	545.5	659.5	2
135	**227**	**Galα1-3 Galβ-sp5 Fucα1-2**	B_{tri}	Btri-C8	658.7	772.7	2
136	**228**	**Galβ1-2Galα1-4GlcNAcβ-sp4**		Ab2aLN	601.6		2
137	**229**	**Galβ1-3Galβ1-4GlcNAcβ-sp4**		Ab3'LN	601.6		2
138	**231**	**Galβ1-4GlcNAcβ1-3GalNAcα–sp3**		LN3Tn	643.6	757.6	2
139	**232**	**Galβ1-4GlcNAcβ1-6GalNAcα–sp3**		LN6Tn	643.6	757.6	2
140	**233**	**Fucα1-4 GlcNAcβ-sp3 Galβ1-3**	Le^a	LeA	586.6	700.6	2
141	**234**	**Galβ1-4 GlcNAcβ-sp3 Fucα1-3**	Le^x	LeX	586.6	700.6	2
142	**235**	**GalNAcα1-3 Galβ-sp3 Fucα1-2**	A_{tri}	Atri	586.6	700.6	2
143	**236**	**GalNAcα1-3 Galβ-sp5 Fucα1-2**	A_{tri}	Atri-C8	699.8		2
144	**237**	**GalNHα1-3 Galβ-$OCH_2CH_2CH_2NHAc$ Fucα1-2**	AB_{tri}	ABtri	586.6		2
145	**238**	**GalNAcβ1-4Galβ1-4Glcβ-sp3**	GA_2, $GgOse_3$	GA2	602.6		2
146	**239**	**No formula available**					2
147	**240**	**$(Glc\alpha 1\text{-}4)_3\beta$-sp4**	maltotriose	(Ga4)3b	560.5	674.5	2
148	**241**	**$(Glc\alpha 1\text{-}6)_3\beta$-sp4**	isomaltotriose	(Ga6)3b	560.5	674.5	2

149	**242**	**GlcNAcα1-3Galβ1-4GlcNAcβ-sp2**		GNa3'LN-C2	629.6		2
150	**243**	**GlcNAcα1-3Galβ1-4GlcNAcβ-sp3**		GNa3'LN	643.6		2
151	**245**	**GlcNAcα1-6Galβ1-4GlcNAcβ-sp2**		GNa6'LN	629.6		2
152	**246**	**GlcNAcβ1-2Galβ1-3GalNAcα-sp3**		GN2'TF	643.6	757.6	2
153	**247**	**GlcNAcβ1-3Galβ1-3GalNAcα-sp3**		GN3'TF	643.6		2
154	**248**	**GlcNAcβ1-3Galβ1-4Glcβ-sp2**		GN3'Lac	588.6		2
155	**249**	**GlcNAcβ1-3Galβ1-4GlcNAcβ-sp2**		GN3'LN-C2	629.6	743.6	2
156	**250**	**GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3**		GN3'LN	643.6	757.6	2
157	**251**	**GlcNAcβ1-4Galβ1-4GlcNAcβ-sp2**		GN4'LN	629.6		2
158	**252**	**GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ-sp4**	chitotriose	Ch3	683.6		2
159	**253**	**GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2**		GN6'LN	629.6		2
160	**254**	**GlcNAcβ1-6** **GalNAcα-sp3** **Galβ1-3**	core 2	core2	643.6	757.6	2
161	**255**	**GlcNAcβ1-6** **GalNAcα-sp3** **GlcNAcβ1-3**	core 4	core4	684.7	798.7	2
162	**256**	**No formula available**					2
163	**258**	**Manα1-6** **Manβ-sp4** **Manα1-3**	Man_3	(Ma)3b	560.5		2
311	**259**	**Galβ1-4** **GlcNAcβ-sp3** **Galβ1-3**		(Ab)2-3,4GN	602.6		2
164	**287**	**Fucα1-4** **GlcNAcβ-sp3** **3-O-Su-Galβ1-3**	Su-Le^a	3'SuLeA	666.7		2
165	**288**	**3-O-Su-Galβ1-4** **GlcNAcβ-sp3** **Fucα1-3**	Su-Le^x	3'SuLeX	666.7		2
166	**289**	**Neu5Acα2-6** **GalNAcα-sp3** **Galβ1-3**	6-SiaTF	6SiaTF	731.7		2
167	**290**	**Neu5Acα2-6** **GalNAcα-sp3** **Galα1-3**		A3a(Sia)Tn	731.7		2

168	**291**	**No formula available**					2
169	**292**	**Neu5Acα2-3Galβ1-3GalNAcα-sp3**	3'-Sia-TF	Sia3'TF	731.7		2
170	**293**	**Neu5Acα2-3Galβ1-4Glcβ-sp3**	3'SL	3'SL	690.7		2
171	**294**	**Neu5Acα2-3Galβ1-4Glcβ-sp4**	3'SL	3'SL-Gly	689.6		2
172	**295**	**Neu5Acα2-6Galβ1-4Glcβ-sp2**	6'SL	6'SL-C2	676.6		2
173	**296**	**Neu5Acα2-6Galβ1-4Glcβ-sp4**	6'SL	6'SL-Gly	689.6		2
174	**297**	**Neu5Acβ2-6Galβ1-4Glcβ-sp2**	β-6'SL	b6'SL	676.6		2
175	**298**	**Neu5Acα2-3Galβ1-4GlcNAcβ-sp3**	3'SLN	3'SLN	731.7		2
176	**299**	**Neu5Acα2-3Galβ1-3GlcNAcβ-sp3**	3'-SiaLe^{c}	3'SiaLeC	731.7		2
177	**300**	**Neu5Acα2-6Galβ1-4GlcNAcβ-sp3**	6'SLN	6'SLN	731.7		2
178	**301**	**Neu5Acα2-6Galβ1-4GlcNAcβ-sp8**	6'SLN	6'SLN-PEG	938.0		2
179	**302**	**Neu5Acβ2-6Galβ1-4GlcNAcβ-sp3**	β6'SLN	b6'SLN	731.7		2
180	**304**	**Neu5Gcα2-6Galβ1-4GlcNAcβ-sp3**	6'SLN(Gc)	6'SLN(Gc)	747.7		2
181	**305**	**Neu5Gcβ2-6Galβ1-4GlcNAcβ-sp3**	β6'SLN(Gc)	b6'SLN(Gc)	747.7		2
182	**306**	**9-NAc-Neu5Acα2-6Galβ1-4GlcNAcβ-sp3**		9NAc-6'SLN	788.8		2
183	**307**	**KDNα2-3Galβ1-3GlcNAc-sp2**	KDN-Le^{C}	KDN-LeC	676.63		2
184	**308**	**KDNα2-3Galβ1-4GlcNAc-sp2**	KDN-LacNAc	KDN-LN	676.63		2
185	**309**	**Neu5Acα2-6 GalNAcα-sp3 Neu5Acα2-3**		Sia2-3,6Tn	882.8 (Na^{+})		2
186	**310**	**3'SiaLacNAcβ-$OCH_2CH_2CH_2NH$-(3'SiaLacNAc-amide-sp3)**		(3'SLN)2	1445.4		2
187	**313**	**4-O-Su-Neu5Acα2-3Galβ1-4GlcNAcβ-sp3**	4''-Su-3'SLN	3'SLN4''Su	833.8 (Na^{+})		2
188	**314**	**9-O-Su-Neu5Acα2-3Galβ1-4GlcNAcβ-sp3**	9''-Su-3'SLN	3'SLN9''Su	833.8 (Na^{+})		2
189	**315**	**Neu5Acα2-3Galβ1-4-(6-O-Su)GlcNAcβ-sp3**	6-Su-3'SLN	3'SLN6Su	833.8 (Na^{+})		2
190	**317**	**Neu5Acα2-3Galβ1-3-(6-O-Su)GalNAcα-sp3**	6-Su-3'SiaTF	3'SiaTF6Su	833.8 (Na^{+})		2
191	**318**	**Neu5Acα2-6Galβ1-4-(6-O-Su)GlcNAcβ-sp3**	6-Su-6'SLN	6'SLN6Su	833.8 (Na^{+})$_t$		2
192	**319**	**Neu5Acα2-3-(6-O-Su)Galβ1-4GlcNAcβ-sp3**	6'-Su-3'SLN	3'SLN6'Su	833.8 (Na^{+})		2
193	**320**	**4-O-Su-Neu5Acα2-3-(6-O-Su)Galβ1-4GlcNAcβ-sp3**	6',4''-Su_2-3'SLN	3'SLN6',4''Su2	935.8 ($2Na^{+}$)		2
194	**321**	**(Neu5Acα2-8)$_3$-sp3**	$(Sia)_3$	(Sia)3	992.9 ($2Na^{+}$)		2
195	**322**	**(Neu5Acα2-8)$_3$β-sp3**	$(Sia)_3$β	(Sia)3b	992.9 ($2Na^{+}$)		2

		Tetrasaccharides					
196	359	Galα1-3 Galβ1-3GlcNAcβ-sp3 Fucα1-2	B (type 1)	Btype1	748.7		2
197	360	Galα1-3 Galβ1-4GlcNAcβ-sp3 Fucα1-2	B (type 2)	Btype2	748.7		2
198	361	Galα1-3 Galβ1-4GlcNAcβ-sp2 Fucα1-2	B (type 2)	Btype2-C2	734.7		2
199	362	Galα1-3 Galβ1-3GalNAcα-sp3 Fucα1-2	B (type 3)	Btype3	748.7		2
200	363	Galα1-3 Galβ1-3GalNAcβ-sp3 Fucα1-2	B (type 4)	Btype4	748.7	862.7	2
201	364	Galα1-3Galβ1-4 GlcNAcβ-sp3 Fucα1-3	αGalLe^{x}	aGalLeX	748.7		2
202	365	No formula available					2
203	366	GalNAcα1-3 Galβ1-3GlcNAcβ-sp3 Fucα1-2	A (type 1)	Atype1	789.8	903.8	2
204	367	GalNAcα1-3 Galβ1-4GlcNAcβ-sp2 Fucα1-2	A (type 2)	Atype2-C2	775.8	889.8	2
205	368	GalNAcα1-3 Galβ1-4GlcNAcβ-sp3 Fucα1-2	A (type 2)	Atype2	789.8		2
206	369	No formula available					2
207	371	Fucα1-4 GlcNAcβ-sp3 Fucα1-2Galβ1-3	Le^{b}	LeB	732.7	846.7	2
208	372	Fucα1-2Galβ1-4 GlcNAcβ-sp3 Fucα1-3	Le^{y}	LeY	732.7	846.7	2
209	373	Galα1-3Galβ1-4GlcNAcβ1-3Galβ-sp3	Galili (tetra)	Galili4	764.7		2
312	374	Galα1-4 Galβ1-4GlcNAcβ-sp3		Aa2-3',4'LN	764.7		2

		Galα1-3					
210	**375**	**No formula available**					2
211	**376**	**Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp4**	LNT	LNT	763.7	877.7	2
212	**377**	**Galβ1-3GlcNAcβ1-3Galβ1-3GlcNAcβ-sp2**		LeCb3'LeC	791.8	905.8	2
213	**378**	**No formula available**					2
214	**379**	**Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3**		LeCb3'LN	805.8	919.8	2
215	**380**	**No formula available**					2
216	**381**	**Galβ1-3GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2**		LeCb6'LN	791.8	905.8	2
217	**382**	**Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-sp3**	Asialo-GM1	aGM1	764.7		2
218	**383**	**Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-sp4**	LNnT	LNnT	763.7		2
219	**384**	**Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp2**	i	LNb3'LN-C2	791.7		2
220	**385**	**Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3**	i	LNb3'LN	805.8		2
221	**386**	**No formula available**					2
222	**387**	**Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2**		LNb6'LN	791.7		2
223	**388**	**Galβ1-4GlcNAcβ1-6 GalNAcα-sp3 Galβ1-3**		LNb6TF	805.8	919.8	2
224	**389**	**GalNAcβ1-3Galα1-4Galβ1-4Glcβ-sp3**	Gb4, P	Gb4	764.7		2
225	**390**	**(Glcα1-4)$_4$β-sp4**	maltotetraose	(Ga4)4b	722.7	836.7	2
226	**391**	**(Glcα1-6)$_4$β-sp4**	isomaltotetraose	(Ga6)4b	722.7		2
313	**394**	**GlcNAcβ1-4 Galβ1-4GlcNAcβ-sp2 GlcNAcβ1-3**		GN2-3',4'LN	832.8		2
314	**395**	**GlcNAcβ1-6 Galβ1-4GlcNAcβ-sp2 GlcNAcβ1-3**	Tk	Tk	832.8		2
227	**396**	**No formula available**					2
315	**397**	**Galβ1-3GlcN(Fm)β1-3Galβ1-4GlcNAcβ-sp3**	formylLe^{C}β3LN	LeC(Fm)b3'LN	791.8		
228	**419**	**3-O-SuGalβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3**		(3'SuLN)3'LN	907.8		2
229	**420**	**4-O-SuGalβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3**		(4'SuLN)3'LN	907.8		2
230	**421**	**GalNAcβ1-4 Galβ1-4Glcβ-sp2 Neu5Acα2-3**	GM2	GM2	879.8		2

231	**422**	**Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ-sp3**		3'SLNb3A	893.9		2
232	**423**	**Neu5Acα2-3Galβ1-4 GlcNAcβ-sp3 Fucα1-3**	$SiaLe^x$	SiaLeX	877.9		2
233	**425**	**No formula available, SleXfuc-beta**					2
234	**426**	**Fucα1-4 GlcNAcβ-sp3 Neu5Acα2-3Galβ1-3**	$SiaLe^a$	SiaLeA	877.9		2
235	**427**	**Fucα1-4 GlcNAcβ-sp4 Neu5Acα2-3Galβ1-3**	$SiaLe^a$	SiaLeA-Gly	876.8		2
236	**428**	**Neu5Acα2-3Galβ1-4 6-O-Su-GlcNAcβ-sp3 Fucα1-3**		SiaLeX6Su	979.9 (Na^+)		2
237	**429**	**Neu5Acα2-3(6-O-Su)Galβ1-4 GlcNAcβ-sp3 Fucα1-3**		SiaLeX6'Su	979.9 (Na^+)		2
238	**430**	**No formula available**					2
239	**431**	**No formula available**					2
240	**432**	**No formula available**					2
241	**433**	**Neu5Acα2-6 GalNAcα-sp3 Neu5Acα2-3Galβ1-3**	Sia_2-TF	Sia2-3',6TF	1044.9 (Na^+)		2
242	**434**	**Neu5Acα2-8Neu5Acα2-3Galβl-4Glcβ-sp4**	GD3	GD3	1002.9 (Na^+)		2
243	**435**	**No formula available**					2
244	**436**	**No formula available**					2
		Penta-nona saccharides					
245	**479**	**Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp4**	LNFP-I	Htype1Lac	909.9	1023.9	2
246	**480**	**Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp2**	H(type 1) penta	Htype1LN	937.91		2
247	**481**	**Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-sp4**	Galili (penta)	Galili5	925.8	1039.8	2
248	**483**	**Galα1-3 Galβ1-4 Fucα1-2 GlcNAcβ-sp3 Fucα1-3**	BLe^y	BLeY	894.9		2
249	**485**	**No formula available**					2
250	**486**	**No formula available**					2

251	**488**	**Galβ1-4GlcNAcβ1-6 GalNAcα-sp3 Galβ1-4GlcNAcβ1-3**		LN2-3,6Tn	1008.9		2
252	**489**	**GlcNAcβ1-6 Galβ1-4GlcNAc-sp2 Galβ1-4GlcNAcβ1-3**		LN3'(GN6')LN	994.9		2
253	**490**	**Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ-sp2 GlcNAcβ1-3**		LN6'(GN3')LN	994.9		2
254	**492**	**$(Glc\alpha 1\text{-}6)_5\beta$-sp4**	isomaltopentaose	(Ga6)5b	884.8	998.8	2
255	**493**	**$(GlcNAc\beta 1\text{-}4)_5\beta$-sp4**	chitopentaose	Ch5	1090.0		2
256	**495**	**Manα1-6 Manα1-6 Manα1-3 Manβ-sp4 Manα1-3**	Man5	(Ma)5b	966.9		2
257	**496**	**Fucα1-4 GlcNAcβ1-3Galβ1-4Glcβ-sp4 Fucα1-2Galβ1-3**	Le^b-Lac	LeBLac	1056.0	1170.0	2
316	**497**	**Fucα1-2Galβ1-4 GlcNAcβ1-3Galβ1-4Glcβ-sp4 Fucα1-3**	Le^Y-Lac	LeYLac	1056.0		2
258	**498**	**$(Gal\beta 1\text{-}4GlcNAc\beta 1\text{-}3)_3$-sp3**	$(LN)_3$	(LNb3')3	1171.1	1285.1	2
259	**499**	**Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAc-sp2 Galβ1-4GlcNAcβ1-3**	I	LN2-3',6'LN	1157.1		2
260	**502**	**$(Glc\alpha 1\text{-}6)_6\beta$-sp4**	maltohexaose	(Ga6)6b	1046.9	1160.9	2
261	**503**	**$(GlcNAc\beta 1\text{-}4)_6$-sp4**	chitohexaose	Ch6	1293.3		2
262	**504**	**$(A\text{-}GN\text{-}M)_2$-3,6-M-GN-GNβ-sp4**	9-OS	9-OS	1697.6		2
263	**505**	**$(GN\text{-}M)_2$-3,6-M-GN-GNβ-sp4**	7-OS	7-OS	1373.3		2
264	**527**	**Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp2**	3'SLN-LacNAc	3'SLN-LN	1083.02		2
265	**528**	**Neu5Acα2-3Galβ1-4 GlcNAcβ1-3Galβ-sp3 Fucα1-3**	$SiaLe^x$-3Gal	SiaLeX3A	1040.0		2
266	**529**	**Neu5Acα2-6 GlcNAcβ1-3Galβ1-4Glcβ-sp4 Galβ1-3**	LSTb	LSTb	1055.0		2
267	**530**	**No formula available $(Neu5Ac\alpha 2\text{-}3Gal\beta 1)_2$-3,4-GlcNAc-sp3**					2

268	**531**	**GalNAcβ1-4 Galβ1-4Glc-sp2 Neu5Acα2-8Neu5Acα2-3**	GD2	GD2	1193.1 (Na^+)		2
269	**532**	**Neu5Acα2-8Neu5Acα2-8Neu5Acα2-3Galβ1-4Glc-sp2**	GT3	GT3	1303.1 ($2Na^+$)		2
270	**533**	**GalNAcβ1-4 Galβ1-4Glc-sp2 $(Neu5Ac\alpha2\text{-}8)_2Neu5Ac\alpha2\text{-}3$**	GT2	GT2	1506.3 ($2Na^+$)		2
		Higher oligosaccharides					
271	**624**	**$(GlcA\beta1\text{-}4GlcNAc\beta1\text{-}3)_8\text{-}NH_2\text{-}ol$**	hyaluronic acid	HyalU-ol	3207.5 ($7Na^+$)		2
272	**626**	**$(Sia2\text{-}6A\text{-}GN\text{-}M)_2\text{-}3,6\text{-}M\text{-}GN\text{-}GN\text{-}NH_2\text{-}ol$**	11-OS (aminoalditol)	11-OS-ol	2247.1 (Na^+)		2
273	**627**	**$(Sia2\text{-}6A\text{-}GN\text{-}M)_2\text{-}3,6\text{-}M\text{-}GN\text{-}GN\beta\text{-}sp4$**	11-OS, YDS	11-OS	2302.1 (Na^+)		2
317	**900**	H-$(Gly)_6$-NH_2 Gly6-amide, linear	Gly6	Gly6	359.3		2
274	**comp 1**	**GlcNAcβ2Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6 GlcNAcβ2Manα3**		NGA2B	1592.62		53.37 nmol
275	**comp 2**	**Galβ4GlcNAcβ2Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6 Galβ4GlcNAcβ2Manα3**		4'-NA2B	1916.79		44.34 nmol
276	**comp 3**	**Neu5Acα3Galβ4GlcNAcβ2Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6 Neu5Acα3Galβ4GlcNAcβ2Manα3**		3'-A2B	2499.30		34.01
280	**comp 4**	**Neu5Acα6Galβ4GlcNAcβ2Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6 Neu5Acα6Galβ4GlcNAcβ2Manα3**		6'-A2B	2499.30		34.01
281	**comp 5**	**GlcNAcβ2Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6 GlcNAcβ4(GlcNAcβ2)Manα3**		NGA3B(2-4)	1795.70		49.03
282	**comp 6**	**Galβ4GlcNAcβ2Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6 Galβ4GlcNAcβ4(Galβ4GlcNAcβ2)Manα3**		4'-NA3B(2-4)	2282.12		38.56
277	**comp 7**	**Neu5Acα3Galβ4GlcNAcβ2Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6 Neu5Acα3Galβ4GlcNAcβ4(Neu5Acα3Galβ4GlcNAcβ2)Manα3**		3'-A3B(2-4)	3155.89		27.88
278	**comp 8**	**Neu5Acα6Galβ4GlcNAcβ2Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6 Neu5Acα6Galβ4GlcNAcβ4(Neu5Acα6Galβ4GlcNAcβ2)Manα3**		6'-A3B(2-4)	3155.89		27.88
279	**comp 9**	**GlcNAcβ6(GlcNAcβ2)Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6 GlcNAcβ2Manα3**		NGA3B(2-6)	1795.70		46.22
286	**comp 10**	**Galβ4GlcNAcβ6(Galβ4GlcNAcβ2)Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6 Galβ4GlcNAcβ2Manα3**		4'-NA3B(2-6)	2282.12		36.37
287	**comp 11**	**Neu5Acα3Galβ4GlcNAcβ6(Neu5Acα3Galβ4GlcNAcβ2)Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6 Neu5Acα3Galβ4GlcNAcβ2Manα3**		3'-A3B(2-6)	3155.89		26.30
288	**comp**	**Neu5Acα6Galβ4GlcNAcβ6(Neu5Acα6Galβ4GlcNAcβ2)Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6**		6'-A3B(2-6)	3155.89		26.30

	12	**Neu5Acα6Galβ4GlcNAcβ2Manα3**					
283	**comp 13**	**GlcNAcβ6(GlcNAcβ2)Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6 GlcNAcβ4(GlcNAcβ2)Manα3**		NGA4B	1998.89		40.02
284	**comp 14**	**Galβ4GlcNAcβ6(Galβ4GlcNAcβ2)Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6 Galβ4GlcNAcβ4(Galβ4GlcNAcβ2)Manα3**		4'-NA4B	2647.46		30.21
285	**comp 15**	**Neu5Acα3Galβ4GlcNAcβ6(Neu5Acα3Galβ4GlcNAcβ2)Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6 Neu5Acα3Galβ4GlcNAcβ4(Neu5Acα3Galβ4GlcNAcβ2)Manα3**		3'-A4B	3812.47		20.98
289	**comp 16**	**Neu5Acα6Galβ4GlcNAcβ6(Neu5Acα6Galβ4GlcNAcβ2)Manα6 GlcNAcβ4Manβ4GlcNAcβ4GlcNAcβ-sp6 Neu5Acα6Galβ4GlcNAcβ4(Neu5Acα6Galβ4GlcNAcβ2)Manα3**		6'-A4B	3812.47		20.98

Each vial contains 2 µl 10 mM oligosaccharide solution (20 nmol of the OS) in 20% (v/v) DMSO in H_2O for 200 µl of the 100 µM solution preparation

Comp # - compounds from Carlo Unvergazt (obtained in October 2009-11-28)

Concentration of the controls

Short name	Controls	Initial concentration	1st conc. or dilution	2nd conc. or dilution
StrCy3	Streptavidin-Cy3 (Invitrogen)	1 mg/ml	50 ug/ml	10 ug/ml
hIgG	human IgG (Sigma)	10mg/ml	500 ug/ml	100 ug/ml
mIgG	murine IgG (Sigma)	1 mg/ml	500 ug/ml	100 ug/ml
FGF23	20-mer peptide-biotin (FGF23) (Blixt)	10 mg/ml	100 ug/ml	20 ug/ml
iM-FITC	2-mer peptide-FITC (iM) (Blixt)	?	1 : 2	1 : 10

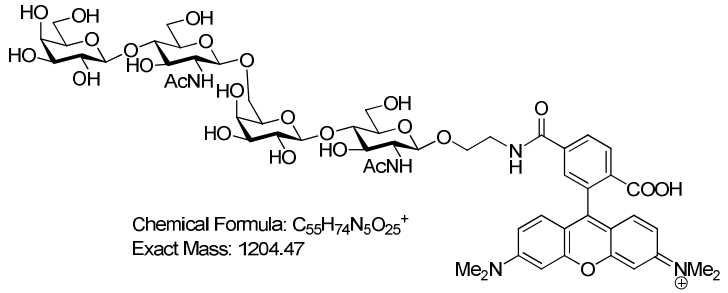
LEGEND:

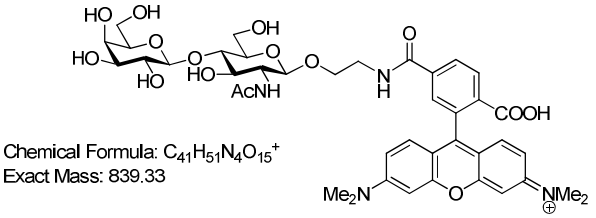
sp2 = C2 = $-O(CH_2)_2NH_2$
sp3 = C3 = $-O(CH_2)_3NH_2$
sp4 =Gly = $-NHCOCH_2NH_2$
sp5 =C8 = $-O(CH_2)_3NH\text{-}CO(CH_2)_5NH_2$
sp6 = Ox = $-N(Me)O(CH_2)_3NH_2$
sp7 = Ph = $-OC_6H_4\text{-}p\text{-}NH_2$
sp8 = PEG = $-(OCH_2CH_2)_6NH_2$
sp9 = Bn = $-OCH_2C_6H_4\text{-}p\text{-}NHCOCH2NH2$

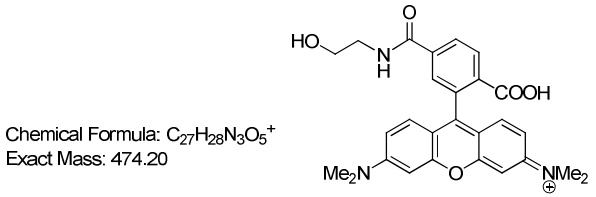
sp0 = other spacers:Asn, Ser, C2Et (see details in the column "Spacered form of saccharide")
Asn = asparagine, NH-CO-$CH_2CH(COOH)NH_2$
Ser = serine, -$OCH_2CH(COOH)NH_2$
C2Et = -$O(CH_2)_2NHEt$

A = Gal
AN = GalNAc
Ch = chito
F = L-Fuc
G = Glc
Gc = glycolyl
GN = GlcNAcβ
R = Rha
i = iso
Lac = lactose
LN = N-acetyllactosamine
M = Man
MN = ManNAc
Malt = maltose
OS = oligosaccharide
P = phosphate
S = Sia = Neu5Acα
Su = sulfate
Tn = GalNAcα
U = uronic acid

Supplementary Fig 1. Structures and molecular weights of LacNAcβ1,6-LacNAc-TMR, and products of chitinase hydrolysis.







A Diverse Range of Bacterial and Eukaryotic Chitinases Hydrolyzes the LacNAc (Gal β1–4GlcNAc) and LacdiNAc (GalNAcβ1–4GlcNAc) Motifs Found on Vertebrate and Insect Cells

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