Exploring the unique N-glycome of the opportunistic human pathogen Acanthamoeba*

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Running title: N-glycans of Acanthamoeba

Capsule:
Background: Acanthamoeba is a facultative parasite of humans capable of causing keratitis or encephalitis.
Results: Novel N-glycan modifications, including core mannosylated fucose, mannose 6-phosphate and multiple pentose residues, were observed.
Conclusion: Acanthamoeba is predicted to possess all ER glycosyltransferases involved in N-glycosylation as well as unusual fucosyl- and pentosyltransferases in the Golgi.
Significance: Examining Acanthamoeba glycosylation may yield insights into virulence factors for this genus of therapeutically-resistant parasites.

Abstract
Glycans play key roles in host-pathogen interactions, thus knowing the N-glycomic repertoire of a pathogen can be helpful in deciphering its methods of establishing and sustaining a disease. Therefore we sought to elucidate the glycomic potential of the facultative amoebal parasite Acanthamoeba. This is the first study of its asparagine-linked glycans, for which we applied biochemical tools and various approaches of mass spectrometry. An initial glycomic screen of eight strains from five genotypes of this human pathogen suggested, in addition to the common eukaryotic oligomannose structures, the presence of pentose and deoxyhexose residues on their N-glycans. A more detailed analysis was performed on the N-glycans of a genotype T11 strain (4RE); fractionation by HPLC and mass spectrometric MS/MS analyses indicated the presence of a novel mannosylfucosyl modification of the reducing-terminal core as well as phosphorylation of mannose residues, methylation of hexose and various forms of pentosylation. The largest N-glycan in the 4RE strain contained two N-acetylhexosamine, thirteen hexose, one fucose, one methyl and two pentose residues; however, in this and most other strains analysed, glycans with compositions of Hex8-9HexNAc2Pnt0-1 tended to dominate in terms of abundance. Although no correlation between pathogenicity and N-glycan structure can be proposed, highly unusual structures in this facultative parasite can be found which are potential virulence factors or therapeutic targets.

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Introduction

*Acanthamoeba* is a unicellular facultative pathogenic amoeba that is the causative agent of two human diseases, namely *Acanthamoeba* keratitis and granulomateous amoebic encephalitis, the latter being nearly always fatal in patients with underlying co-morbidities. *Acanthamoeba* keratitis is nowadays commonly associated with the wearing of contact lenses. Indeed, although high amoebal densities brought into the eye are more common but not limited to individuals wearing contact lenses, contact lens wearers often have microlesions in the cornea, which facilitate the invasion of the amoebae into deeper layers. The potential for affecting humans is intensified due to its ubiquitous presence in various environmental sources from which the amoeba can be isolated, including public water supplies, bottled water and the atmosphere (1,2). *Acanthamoeba* trophozoites are capable of encystment, a process triggered by harsh environmental conditions during which a double cyst wall is formed and metabolism is reduced to form dormant cysts, which often renders treatment of *Acanthamoeba* keratitis ineffective. Furthermore, the denomination of species in the *Acanthamoeba* genus was highly disputed as it was based on morphological details subject to variability depending on environmental factors (3); therefore, a system of twelve genotypes was established in 1996 (4), which has meanwhile been expanded to 17 genotypes (5,6). The high genetic variability of the genus is reflected by the fact that no single strain originates either from multiple patients (unless family members) or from more than one environmental source.

A vital step in the process of invading a host is often the attachment of parasites to epithelia, which in several cases is associated with lectin-oligosaccharide interactions. Examples of such include the host-pathogen interactions of other protozoa, including the dysentery-causing obligate parasite *Entamoeba histolytica* and sexually-transmitted *Trichomonas vaginalis*. Indeed a lectin-based invasion mechanism is known for *E. histolytica*, specifically involving attachment to the colonal epithelia via a glycosylated Gal- and GalNAc binding lectin (7), whereas in the case of *T. vaginalis*, epithelial host galectin-1 binds to the lipoglycan of the parasite (8). In the case of the establishment of *Acanthamoeba* keratitis, a potentially glycosylated mannose binding lectin of the parasite mediates attachment to the host cornea (9).

In this study we screened the N-glycomic potential of eight strains, including four clinical isolates, representing five genotypes of the genus *Acanthamoeba*, prior to more in-depth analyses on one strain (4RE). Although there are various earlier reports on a partial characterization of lipophosphonoglycan (10) and the presence of a *N*-acetylglucosaminyl-1-phosphotransferase (11) as well as various radiolabelling (12), lectin binding (12-15) or monosaccharide composition studies (16), our present data are the first regarding the actual structures of glycosylated macromolecules of this genus. As it has been shown that many protozoal parasites synthesise highly unique N-glycans (17), it can be hypothesised that the investigation of
the N-glycosylation of parasitic and non-parasitic *Acanthamoeba* strains may yield insights into virulence factors.

**Experimental Procedures**

*Cultivation of amoebae:* Axenic cultures were maintained in proteose peptone-yeast extract-glucose medium (PYG) containing antibiotics and antifungicotics (18) at 26°C. Trophozoites were collected via centrifugation and washed with PBS buffer. All cultures were examined by light microscopy to verify the absence of contamination or of endocytobionts. The strains used (see also Table I and Ref. (19)) in the glycomic screen are as follows: 4RE (genotype T11, ATCC PRA-115) (20), 1BU (genotype T4, ATCC PRA-113) (21), ATCC 30234 (genotype T4) (22), Neff (genotype T4, ATCC 30010) (23), PAT06 (genotype T4) (24), 72/2 (genotype T5, ATCC 50705) (21), 11DS (genotype T6, ATCC PRA-112) (20) and Pb30/40 (genotype T7, ATCC PRA-287) (25).

**Western Blotting:** Cells (approximately 10^7) were mixed with 400µl of 2-fold concentrated reducing Laemmli buffer and incubated at 95°C for 10 min. The samples were centrifuged, aliquots were separated by SDS-PAGE and transferred to nitrocellulose using a semi-dry blotting apparatus. After blocking with 0.5% (w/v) bovine serum albumin in Tris-buffered saline, the membranes were incubated for 1 hour with either rabbit anti-horseradish peroxidase (anti-HRP, Sigma-Aldrich; 1:10000) or biotinylated lectins (Vector Laboratories; 1:2000) from *Aleuria aurantia* (AAL), *Erythrina cristagalli* (ECL), *Lens culinaris* (LCA), *Ricinus communis* (RCA) *Triticum vulgaris* (wheat germ; WGA) and *Vicia villosa* (VVA) in blocking buffer; after washing, alkaline phosphatase conjugated forms of either goat anti-rabbit or streptavidin (1:2000) were used with subsequent colour detection with SigmaFAST™ 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. 6-Phosphorylation of mannose was detected using a myc-tagged single chain antibody fragment (scFv M6P-1, 5 µg/ml), followed by detection with monoclonal anti-myc antibody (9E10, Sigma-Aldrich; 1:5000) and enhanced chemiluminescence (Pierce) (26); as a positive control, 20 µg of an extracts from *Acp2/Acp5*−/− mice deficient in two lysosomal acid phosphatases, prepared as described previously (27), were co-blotted.

**Glycan Preparation:** N-glycans were prepared from axenically grown *Acanthamoeba* by enzymatic release of glycans from partially-purified glycopeptides using either PNGase A or F (Roche) as previously described for *Dictyostelium* (28). Generally, cells were taken up in formic acid (up to 5%) denatured at 100°C for 10 minutes and lysed using a glass homogeniser; the lysate was then subject to proteolysis overnight using 1 mg of pepsin per gram wet weight of cells. Thereafter the proteolysate was applied to Dowex 50 cation-exchange resin and material eluted with 0.5 M ammonium acetate (pH 6) was desalted (Sephadex G25) prior to addition of either PNGase A or F (Roche). The released N-glycans, which did not bind a second Dowex 50 column, were pyridylaminated overnight prior to gel filtration (Sephadex G15) (29); further analysis by MALDI-TOF MS and HPLC was performed as described below.

**Glycan Analysis:** The N-glycome of the 4RE strain was fractionated by either reversed-phase HPLC (Agilent Hypersil ODS, 4 × 250 mm; gradient of 0.3% methanol per
minute using 0.1 M ammonium acetate, pH 4, as buffer; 1.5 ml/min) or normal-phase HPLC (Tosoh TSKgel Amide-80, 4.6 × 250 mm; gradient from 71.25% to 61.75% acetonitrile over 20 minutes followed by a gradient from 61.75% to 47.5% acetonitrile for a further 45 minutes using 0.01M ammonium formate, pH 7, as buffer; 1 ml/min). Glycans were respectively detected by fluorescence with excitation/emission wavelengths of 320/400 in the case of RP-HPLC or 310/380 nm in the case of NP-HPLC (29,30). Selected fractions were subject to two-dimensional HPLC with normal-phase HPLC in the first dimension and reversed-phase HPLC in the second. Both columns were calibrated in terms of glucose units using a pyridylaminated dextran hydrolysate standard; comparisons were also made with the elution times of pyridylaminated Man$_3$GlcNAc$_2$ and Man$_9$GlcNAc$_2$ (Takara) as well as the endoplasmic reticulum mannosidase I digestion product (Man$_9$GlcNAc$_2$) of the latter. For comparative examination of the properties of a β1,2-xylosylated plant glycan (Man$_3$GlcNAc$_2$Xyl), a relevant standard was isolated after PNGase F digestion of an pepsinised extract of white beans. Further analysis by MS was performed after treatment overnight with 0.2 µl of either jack bean α-mannosidase (Sigma-Aldrich) and/or bovine α-fucosidase (Sigma-Aldrich) or recombinant endoglycosidase H (Roche) or Aspergillus α1,2-mannosidase (Prozyme) in 50 mM ammonium acetate buffer, pH 5, at 37 °C. Monoisotopic MALDI-TOF-MS was performed using a Bruker Ultraflex or Autoflex Speed TOF-TOF instrument with 2,5-dihydroxybenzoic acid (DHB) or 6-aza-2-thiothymine (ATT) as matrix; MS/MS was performed by laser-induced dissociation. Spectra were processed with the manufacturer’s software (Bruker Flexanalysis 3.3.80) using the SNAP algorithm with a signal/noise threshold of 6 for MS (unsmoothed) and 3 for MS/MS (four-times smoothed). Thereafter, mass spectra were analysed manually; after an initial assessment of each entire spectrum on the basis of known glycan masses and putative glycan series, each individual HPLC-purified fraction was subject to MS and MS/MS to determine the presence of key fragments (e.g., those corresponding to core fucose, pentosylated and methylated residues) and fragment series. These manual analyses validated the proposed calculated composition and allowed the prediction of structures, which in part could be further verified by exoglycosidase digestion or HPLC elution times. For semi-quantitative comparison, the relative intensities of the ten most abundant glycans from each strain were calculated with reference to the MS peak intensity of the Hex$_9$HexNAc$_2$ glycan (set as 100).

Permethylation of the major 2D-HPLC N-glycan was achieved by a modification of the “Methylation with Sodium Hydroxide and Methyl Iodide in Dimethyl Sulfoxide with the Presence of Water” protocol from Ciucanu and Costello (31). Dry glycans were dissolved in dimethyl sulphoxide with a trace of water; after addition of powdered NaOH dissolved in dimethyl sulphoxide and subsequent shaking, deuterated iodomethane (Sigma-Aldrich) was added to a final concentration of 3 mM and the mixture was incubated at RT for 10 min. The liquid phase was transferred to a fresh reaction tube where the reaction was quenched with water and neutralized with 0.1 M HCl. Isolation of the perdeuteromethylated products was achieved by repeated extraction with chloroform. The sample was taken up in 50% methanol in 1 mM sodium acetate and 2,5-dihydroxybenzoic acid was used as matrix for MALDI-TOF-MS and MS/MS analysis.
Results

Glycomic screening of eight Acanthamoeba strains: In order to examine the glycomic potential of Acanthamoeba, we initially tested cell extracts of trophozoites of eight selected strains (see Table 1) by Western blotting with a range of antibodies and lectins. From these blots we concluded that Acanthamoeba is able to synthesize glycosidic structures with varying monosaccharide composition. In particular, we first investigated the ability of its proteins to bind to anti-horseradish peroxidase (anti-HRP), an antibody raised against a common plant glycan structure (32), which recognises epitopes in a range of plant, invertebrate and protist organisms (33). Positive results from the anti-HRP blots led to the conclusion, that most of the selected strains synthesize either core α1,3-fucose and/or β1,2-xylose epitopes on glycoproteins across the molecular mass range (Figure 1A). Reactivity to fucose-recognising lectins AAL and LCA, a GalNAc-recognising lectin VVA and galactose-recognising lectins ECL and RCA was also widespread amongst the strains, whereas generally trace reactivity towards WGA was observed (Supplementary Figure 1). Furthermore, reactivity to a single-chain antibody specifically recognising mannose 6-phosphate residues was apparent particularly towards proteins of less than 50 kDa (Figure 1B). To gain further insight into the N-glycosylation potential of these Acanthamoeba strains, it was therefore necessary to isolate the protein-bound N-glycans from cell pellets and use chromatographic and mass spectrometric means of analysis.

MALDI-TOF MS and MS/MS of N-glycans released by PNGase F was employed to screen the eight strains for the range of structures present; these data, summarized in Table 2 and Supplementary Figure 2, suggest that Acanthamoeba is capable of producing N-glycans not previously found in protozoans. The major structures in most strains are large mannosidic glycans with seven to ten hexoses with or without a pentose residue (Hex7−10HexNAc2Pnt0−1). Little difference was apparent when comparing PNGase A and F digests in terms of the degree of modification with deoxyhexose residues (Supplementary Figure 2); furthermore, monosaccharide composition data indicated the presence of xylose and fucose (data not shown). Therefore, we conclude that the pentose residues are, in part, β1,2-xylose residues capable of binding anti-HRP, whereas the deoxyhexose residues are assumed to be, in part, core α1,6-fucose residues. Other than varying amounts of larger glycans (m/z 2200-3000) of differing composition, strains of the genotype T4 (ATCC 30234, Neff, IBU and PAT06) exhibit similar glycomic profiles. The glycan profile of strain 11DS shows large similarity to those of genotype T4 strains, which correlate with the fact that this T6 strain exhibits many traits associated with genotype T4 (20). Analysis of strain 72/2 (genotype T5), the only group III strain tested, revealed similar N-glycosylation as the T4 strains, with a shift to larger structures. A completely different pattern was detected for strain Pb30/40 (genotype T7, morphological group I), which synthesises glycans of smaller mass with the composition Hex5−7HexNAc2Pnt0−5. These characteristics render Pb30/40 unique amongst all eight trophozoites samples examined and fits very well to the fact, that group I is only very distantly related to group II and group III (34). The overall profile of the glycans from group II strain 4RE (genotype T11) revealed close similarity to the T4 strains, with the exception of additional rather large structures, ranging in mass from 2400 Da to
3100 Da, containing ten to thirteen hexoses, two pentoses and maximally one fucose and/or methyl groups. Due to these peculiarities but also the relative simplicity of its glycans in terms of numbers of non-hexose residues, the PNGase F-released glycans of this strain was chosen for further analysis.

Complete N-glycome digestions: The complete pyridylaminated N-glycan pool of the 4RE strain was subject to various enzymatic treatments prior to MALDI-TOF MS (Figure 2). Jack bean α-mannosidase, *Aspergillus* α1,2-mannosidase and endoglycosidase H were employed. Jack bean mannosidase digestion resulted in a significant shift in the N-glycome with the smallest glycans being Hex1HexAc2Pnt0-1 (m/z 665 and 797); especially the glycans of up to Hex0HexNAc2Pnt1 were affected by this treatment, whereas the largest glycans of between 2200 and 3100 Da were rather resistant and maximally one hexose was removed from such large glycans (e.g., from the largest glycan of m/z 3056). Endoglycosidase H removed all oligomannosidic glycans but not pentosylated ones; *Aspergillus* α1,2-mannosidase had, like the jack bean mannosidase, a significant effect, resulting in a large increase in Hex8HexNAc2, compatible with the presence of three or four α1,2-linked mannose residues on Hex9GlcNAc2, but had no effect on the larger glycans. In the case of other strains, combined jack bean mannosidase and endoglycosidase digestion results in similar shifts in the overall spectra, but again the largest glycans appeared to be also generally unaffected (Supplementary Figure 3) suggestive of the presence of terminal residues other than mannose (e.g., glucose, methylated hexose or pentose). For further structural information, the pyridylaminated N-glycans of the 4RE strain were fractionated by normal and reversed-phase HPLC (Supplementary Figures 4 and 5) prior to further mass spectrometric analyses.

Oligomannosidic glycans: Amongst the common, but not most abundant, N-glycans of the 4RE strain are a series of endoglycosidase and mannosidase-sensitive structures (Hex5-9HexNAc2); apparently these can be mainly digested down to a Man1GlcNAc2 stub with m/z 665 as seen in the overall jack bean mannosidase-treated profile or to Man5GlcNAc2 (m/z 1313) with the fungal α1,2-specific enzyme (Figure 2). The putative Man5GlcNAc2 species elutes at around 5 glucose units on RP-HPLC, just prior to the putative Man9GlcNAc2 (Supplementary Figure 4B); this elution order, comparable to literature values (35) and observed when analysing Man9GlcNAc2 and its endoplasmic reticulum α-mannosidase I digestion product (36), would be compatible with the major Man9GlcNAc2 isomer being Man8B. The putative Man5GlcNAc2 in the untreated samples eluted at 7.2 g.u. (Supplementary Figure 4B), thereby co-eluting with a commercial standard and indicating that this glycan corresponds to the normal isomer resulting from Golgi processing.

Core fucosylation and hexose capping of fucose: Amongst the range of glycans from the 4RE strain whose mass indicated the presence of deoxyhexose residues, one of the least complex is Hex6HexNAc2Fuc1 (m/z 1621 as [M+H]+). In addition, fucose residues were found attached to glycans with additional pentoses and methyl groups (see Table 2 for details). Many fucosylated structures from the 4RE strain gave rise not to the m/z 446 ion (GlcNAc1Fuc1-PA) during fragmentation, but to one of m/z 608 (Figure 3A). This fragment indicates a hexose being bound to the core fucose. The Hex9HexNAc2Fuc1 glycan (released with
PNGase F) was sensitive to jack bean α-mannosidase treatment, which resulted in loss of up to five hexose residues (smallest product being of m/z 811) and, upon MS/MS of three of the products, a loss of the m/z 608 fragment ion accompanied by a dominance of an m/z 446 fragment indicative of core fucosylation (Figure 3B shows the MS/MS of the m/z 973 product). Fucosidase treatment had no effect unless preceded by incubation with α-mannosidase; a number of products, the smallest of which is of m/z 665, lacked any MS/MS hallmarks of fucosylation as judged by the presence of the m/z 300 fragment (Figure 3C). On the other hand, Aspergillus β-galactosidase had no effect on this glycan (data not shown). Under the assumption that the jack bean mannosidase contains no further hexosidase activities, these data would indicate that the structure with m/z 1621, based on a typical Man5GlcNAc2 glycan, is decorated with a core α1,6-fucose capped with an α-linked mannose. Similar data on the mannosidase sensitivity of the core hexosylfucose motif were obtained for Hex6HexNAc2Fuc1 from the ATCC 30234 strain. A number of larger glycans from some Acanthamoeba strains are also predicted to carry such a unique epitope (see below).

Phosphorylation of mannose residues: The reactivity of Acanthamoeba lysates with the scFV M6P-1 antibody (Figure 1B) directed us to examine for the presence of potentially phosphorylated glycans in the 4RE strain. A phosphorylated hexose would give rise to a fragment of m/z 243 in positive-ion mode; this fragment, in combination with two other potentially phosphorylated fragments of m/z 405 (Hex2Phos) and 1069 (Hex3HexNAc2Phos-PA), was found when analysing a glycan of m/z 1717. Thus, this species has a predicted composition of Hex7HexNAc2Phos-PA (Figure 4).

Methylation of hexose residues: MS/MS of a number of glycans isolated from the 4RE strain revealed fragments of m/z 177 and 339, which are suggestive of methylation of hexose residues. Examples of methylated glycans include pentosylated and fucosylated structures, including higher molecular weight glycans of the form Hex10-13HexNAc2Pnt2Fuc0,1Me. Other example fragments include m/z 542 and 841 (Hex2HexNAc1Me1 and Hex2HexNAc2Me1-PA) as shown here for glycans with the composition Hex8HexNAc2Me1 and Hex9HexNAc2Pnt2Me (Figure 5). Based on these fragments, we presume that the methylated residue in these cases is one of the α-linked mannose residues attached directly to the core β-mannose.

Structural analysis of pentosylated glycans: While in other strains a greater degree of pentosylation is possible (Table 2), maximally two pentose residues were observed in the glycans of the 4RE strain. The major structures (Hex8-10HexNAc2Pnt1) are assumed to be novel as compared to the known literature; bearing in mind that all examined glycans of this composition elute in multiple RP-HPLC peaks, we conclude that Acanthamoeba is capable of synthesizing several isomers of these structures. Various putatively pentosylated fragments, such as m/z 295 (HexPnt), 336 (HexNAcPnt), 635 (HexNAc2Pnt-PA), 797, 959, 1121 and 1283 (Hex1,4HexNAc2Pnt-PA) were detected. These could be indicative of the presence of pentose close to or in the core region, but are ambiguous; thereby, pentose could in theory be either on the β-linked mannose of the glycan core, as found in plants (which present fragments of m/z 797 and 959; see below), or on the second GlcNAc of the chitobiose unit, as found on N-glycans of T. vaginalis (which present fragments of m/z 336 and 635 (29)), or on a terminal α-mannose as in a
microalga (37); rearrangements during fragmentation can also not be ruled out (38). This large degree of ambiguity compounded by the presence of many isomers makes it difficult to arrive at definitive structural propositions. However, the MS/MS spectrum of a glycan of HexαHexNAc2Pnt2Me (Figure 5B) shows the presence of a pentosylated fragment containing a methylated hexose (m/z 674; Hex2HexNAc1PntMe) as well as a fragment of m/z 797 (HexHexNAc2Pnt-PA), but not of m/z 635 (HexNAc2Pnt-PA); these data suggest strongly that the xylose is linked to the β-linked mannose of this particular glycan.

The most dominant quasimolecular ion in the whole glycome spectrum of 4RE is of m/z 1931 in its protonated form, corresponding to Hex3HexNAc2Pnt1-PA. Species of this composition are enriched in the NP-HPLC fraction IX and were further purified by RP-HPLC. Digestion of the resulting major 2D-purified glycan with jack bean mannosidase resulted in a ladder of products (compare Figures 6A and B). MS/MS of the untreated glycan and its products revealed a fragment of m/z 635 which would be compatible with a composition of HexNAc2Pnt1-PA (Figure 6 C and D) and differs from the fragmentation pattern of an authentic Man3GlcNAc2Xyl1 glycan from beans (Figure 6 E). Perdeuteromethylation of the 2D-HPLC-purified Hex8HexNAc2Pnt1-PA resulted in ions of m/z 2510 and 2527 as well as their sodium adducts 2532 and 2549 (Figure 6F).

It appears that, in this and other perdeuteromethylation experiments, the pyridylamino function may be either mono- or dipermethylated, resulting in a major fragment of m/z 367 and a minor one of m/z 384; furthermore, a fragment of m/z 787 would be compatible with a perdeuteromethylated form of HexNAc2Pnt1-PA and that of m/z 2143 would result from loss of the reducing-terminal GlcNAc-PA (Figure 6G). As this form of Hex8HexNAc2Pnt1 is the dominant structure in 4RE, addition of pentose to the distal core GlcNAc is the major form of pentosylation in this strain, while xylosylation of the core mannose is relatively rare.

Analysis of larger N-glycans: Larger glycans containing xylose and often deoxyhexose and/or methyl residues are also found in the 4RE strain (Table 2). MS/MS of 2D-HPLC purified fractions of these glycans suggested the presence, in many cases, of hexosylated core fucose as judged by the presence of the m/z 608 fragment (Figure 7A-F), similar to the case of the HexαHexNAc2Fuc glycan described above. Two of the largest structures, occurring in the same normal-phase HPLC fraction (XIV), had predicted compositions of Hex12HexNAc2Pnt1Fuc1 and Hex13HexNAc2Pnt2Fuc1Me (m/z 2858 or 3034 as [M+H]+). Attempts at digesting these glycans with glycosidases were largely unsuccessful, which could be due to putative terminal glucosylation or pentosylation as well as steric hindrance; however, a mannosidase-mediated unveiling of the core fucose was observed for these glycans, as shown by the loss of the m/z 608 MS/MS fragment and its replacement by one of m/z 446. Subsequent fucosidase digestion resulted, in turn, in the loss of the m/z 446 fragment and the appearance of a strong one of m/z 300 (Figure 7G,H,I).

Multiple pentosylation in a non-pathogenic strain: Glycans of the non-pathogenic Pb30/40 strain, the only example here of a group I Acanthamoeba, were of interest due to the high degree of pentosylation and the apparent lack of fucose and methyl groups. Indeed, up to five pentose residues on a Man7GlcNAc2 structure are postulated;
complete glycome digestion suggested that a number of mannose residues could be released from such glycans to yield Hex$_4$HexNAC$_2$ (Supplementary Figure 3). Therefore, the question was as to how four or five pentose residues could be attached to a putative Man$_4$GlcNAc$_2$ ‘core’. From the MS/MS data of a purified form of one of these glycans (Figure 8), we assume that two pentose residues are associated with the chitobiose core and that two are more peripheral. Considering that a neutral loss of 299.5 (GlcNAc-PA) was observed from the Hex$_7$HexNAc$_2$Pnt$_4$ glycan and that the m/z 767 (putatively HexNAc$_2$Pnt$_2$-PA) fragment was slightly more abundant than the m/z 563 fragment (putatively HexNAc$_1$Pnt$_2$-PA), we assume that two pentose residues may be associated with the distal (second) core GlcNAc, but that some degree of rearrangement during MS/MS occurs to result in artefactual pentosylation of the reducing-terminus. The traces of potential dipentose-containing small fragments (Pnt$_2$, HexPnt$_2$ and HexNAcPnt$_2$; m/z 265, 427 and 468) would be compatible with modifications on an internal GlcNAc and on a mannose. As five mannose residues were removed by jack bean α-mannosidase and two by Aspergillus α1,2-mannosidase from the purified glycan, we assume the peripheral dipentosylation is present on an inner mannose, but is not directly attached to the core β-mannose.

Various modifications of the core GlcNAc in pathogenic strains: During our survey, it became obvious that many strains express N-glycans with fragments characteristic of core fucosylation. Depending on the strain, fragments of m/z 446 (HexNAcFuc-PA), 608 (HexHexNAcFuc-PA) and 740 (HexHexNAcPntFuc-PA) were observed. The modification of a glycan from 4RE with a mannosylfucosyl motif (indicated by a m/z 608 fragment) has been described above; however, for some strains only an m/z 446 fragment was observed, but in other cases one of m/z 740 is apparent (Figure 9). However, as both non-pathogenic strains such as 4RE (Figure 3) and ATCC 30234 (data not shown) and pathogenic strains such as 11DS, 1BU, 72/2 and PAT06 contain different degrees of capping of core fucose, there is no apparent correlation between the core modifications and pathogenicity. Nevertheless, the HexHexNAcPntFuc-PA core is particularly novel and was only present in the group II strains.

Discussion

The glycomic potential of Acanthamoeba: The genus Acanthamoeba presents an interesting target for glycan analysis for several reasons; one being that the N-glycomes of the only two other amoebozoans studied so far (E. histolytica, D. discoideum) have revealed novel features (28,39). The facultative nature of the pathogenicity of Acanthamoeba sets it apart from Entamoeba, an obligate parasite, and Dictyostelium which is non-parasitic. Thus, glycomic data on Acanthamoeba are of both phylogenetic and biomedical interest. Although a high abundance of larger structures in the pathogenic morphological group III strain 72/2 was detected, significant quantities of large glycans are also observed in both pathogenic (11DS and 1BU) and non-pathogenic group II strains (4RE and ATCC 30234). On the other hand, the glycomes of the pathogenic PAT06 and non-pathogenic Neff, also belonging to group II, are more dominated by unmodified or monopentosylated oligomannosidic glycans. The rather simple glycome of the P30/40 strain, featuring dipentose motifs, is in keeping with it belonging to group I, which is phylogenetically rather distant, in terms of 18S rDNA sequences, to the groups
II and III (40). Thereby, the glycan structures found are indeed novel and unlike those in other amoebae, but there is no obvious strong link with genotype or pathogenicity.

Both familiar and unfamiliar glycan modifications: The mass spectrometric and blotting data did suggest the presence of two mammalian-type glycan modifications: mannose-6-phosphate and core α1,6-fucose. In the 4RE strain, chosen here for further characterisation, one glycan of low abundance was found to present a series of fragments suggestive of phosphorylation (m/z 243, 405); indeed, previous data has shown that Acanthamoeba lysates display UDP-N-acetylglucosamine:glycoprotein N-acetylglucosamine-1-phosphotransferase activity (41). Many of the glycans were core fucosylated as indicated by the fragment of m/z 446 before or after exoglycosidase digestion. These glycans were released by PNGase F and so are not of the core α1,3-fucosylated type found in Dictyostelium (28); together with the sensitivity of this core fucose to bovine α-fucosidase, reactivity to LCA and detection of a relevant fucosyltransferase activity (data not shown), our data indicate that the fucose is α1,6-linked to the reducing terminus.

Although the mammalian type of core fucosylation is present, a number of Acanthamoeba N-glycans carried hexose residues attached to the core fucose (fragment of m/z 608); we initially assumed the epitope in Acanthamoeba to be of the same nature (galactosylation of core fucose) as that found in nematodes, cephalopods, a gastropod and a planaria (30,42-44). This Gal-Fuc epitope confers sensitivity towards a toxic fungal lectin (CGL2) in C. elegans (45) and it is of special interest that Acanthamoeba ATCC 30234 is also sensitive to this lectin (46). However, our analyses indicate that the core fucose is uniquely capped with α-linked mannose. Thereby, the basis for the toxicity of the CGL2 towards Acanthamoeba may be a broader specificity of this lectin than previously thought.

Acanthamoeba synthesizes novel pentosylated structures, which are extremely difficult to characterize given their uncommon nature and their frequently low abundance. Monopentosylated oligomannosidic N-glycans, reminiscent in terms of composition with some structures recently observed in the pathogenic fungus Cryptococcus neoformans (47), are very common in the investigated Acanthamoeba strains and are often the single most abundant N-glycan synthesized, but probably occur as multiple isomers. Based on MS/MS data, we hypothesised that the pentose is most often attached to the second GlcNAc of the core due to the significant occurrence of a fragment of m/z 635; this is also verified by analysis of a perdeuteromethylated structure. This location for pentosylation has been observed in the protist Trichomonas and the microalga Porphyridium (29,37). Only for a methylated glycan could we see clear evidence for a pentosylation of the core β-mannose in the 4RE strain, which would be the location compatible with the anti-HRP cross-reactivity towards amoebal proteins (48). Therefore, we conclude that xylosylation of the β-mannose may be at a relatively low level in Acanthamoeba or that other pentosylated structures cross-react with anti-HRP.

In addition to the monopentosylated glycans, a variety of multiply-pentosylated structures were observed. These are of a very diverse nature with predicted compositions ranging from Hex₂HexNAc₂Pnt₂ to Hex₈HexNAc₂Pnt₆Fuc₁Me. Taking all the
pieces of evidence together we assume that *Acanthamoeba* is capable of transferring pentose residues to the α-linked peripheral mannosas as in microalgae (37); the amount of the glycans available that carry more than one pentose limits the possibilities for their investigation, but we present evidence for dipentose motifs in the Pb30/40 strain. Also, glycans displaying pentose bound to the hexosylfucosyl motif were detected in some strains, but not in the 4RE strain.

The occurrence of methyl groups leads to *Acanthamoeba* N-glycans that bear resemblance to structures previously described for mollusks and some other species. Possibly *Acanthamoeba* is capable of synthesizing methylated N-glycans displaying similarities to those described for mollusks, a planarian and a microalga. *Biomphalaria* for example is capable of attaching 3-O-Me to either of the mannoses that are attached to the β-linked mannosae in glycans carrying a core xylose (49), while *Lymnaea* only methylates the α1,3-linked mannosae (50). The planarian *Dugesia japonica*, on the other hand, modifies terminal mannosae with methyl groups (44). In the microalga *Porphyridium*, internal mannosae residues of glycans, with the composition Hex7-9HexNAc2Pnt0-2Me3, were found to be methylated (37). A glycan of the composition Hex3HexNAc2Me1 from strain 72/2 is reminiscent of a structure of the same composition found in the clam *Hippopus hippocus*; the clam glycan carries a 6-methylated α1,6-linked terminal mannosae residue (51). Most methylated glycans in *Acanthamoeba* are pentosylated and fucosylated; however, the exact nature and location of the methyl groups in *Acanthamoeba* still needs to be determined and leaves plenty of work for further studies.

*Biosynthesis of N-glycans in amoebae: All Acanthamoeba* strains assessed produce comparatively large structures, apparently based on the common oligomannosidic eukaryotic structures Man7-9GlcNAc2 and containing up to four α1,2-mannose residues. Maximally thirteen hexose residues, one of which is attached to a core fucose, were present in the largest glycans, but hardly any glycans smaller than Hex4HexNAc2 are detected. Therefore, in terms of N-glycan precursor assembly, *Acanthamoeba* is possibly closer related to organisms such as yeast, slime moulds, animals and plants than to other parasitic protozoans. Samuelson et al. (52) hypothesised that the protozoans which were obligate parasites, such as *Entamoeba histolytica*, *Trichomonas vaginalis* and trypanosomatids, probably lost different glycosyltransferases required for N-glycan precursor assembly in the course of evolution. This can make sense as obligate parasites tend to be genetically reduced and dependent on the host, whereas a free-living *Acanthamoeba* does not require a host and is highly biochemically autonomous.

tBLASTn searching of whole genome shotgun sequences from the Neff strain (data not shown) suggests that all fourteen standard *alg* genes, including those encoding the complete set of ER mannosyl- and glucosyltransferases, are present in *Acanthamoeba*. Post-transfer, glucosidase and mannosidase activities are also predicted based on the structural and genomic evidence, although the significant amounts of Hex10-12HexNAc2 suggest that deglucosylation in endoplasmic reticulum is incomplete, but the lack of a commercially-available glucosidase II has limited our possibilities of proving that the glucose ‘caps’ are indeed present.

In contrast to the apparent dominance of oligomannosidic glycans, no complex or hybrid type glycans were detected. These findings led us to believe, that
Acanthamoeba lacks GlcNAc-transferase I (GlcNAc-T1) since its activity is necessary for the formation of complex glycans in eukaryotes (53). This would be similar to the case of Dictyostelium, where the GlcNAc-T1 is also thought to be absent (28) and it has been hypothesised that a classical GlcNAc-T1 is also probably absent in trypanosomatids and trichomonads (29). On the other hand, tBLASTn results suggest that the Neff strain genome may encode proteins with homology to characterised GlcNAc-T1 and Golgi mannosidase II proteins (data not shown). Nevertheless, both a xylosyltransferase and a fucosyltransferase activity were detected by us, which accept Man$_5$GlcNAc$_2$ as a substrate (data not shown) and so, therefore, are GlcNAc-T1-independent.

**Conclusion:** Our data would indicate that Acanthamoeba is the third amoebozoan (Entamoeba and Dictyostelium also belonging to this group) to express unusual N-glycans. These three organisms are the only amoebozoans from which extensive data on N-glycans is available up to date. It is therefore still too early to draw conclusions about this entire group of organisms; elucidating the glycomic potential of further amoebozoans though has the potential to yield new insights into variations of the common eukaryotic N-glycosylation pathway. We further predict that Acanthamoeba utilizes the common eukaryotic pathway for N-glycan synthesis, as it is apparently capable of synthesizing the complete lipid-linked oligosaccharide precursor dolichol-PP-Man$_9$GlcNAc$_2$, which can be deduced from the presence of large oligomannosidic structures such as Man$_9$GlcNAc$_2$ and the putatively glucosylated Hex$_{10-12}$HexNAc$_2$-based species, as well as the presence of all standard alg genes in its genome. This trait might be a distinguishing factor between obligate and facultative protozoal pathogens. In addition there must be novel genes encoding xylosyltransferases and fucosyltransferases or homologues of already characterized enzymes of this type which display altered substrate specificity. Taken together these data present Acanthamoeba as a potentially pathogenic organism capable of synthesizing a large number of highly uncommon N-glycans. Certainly, these glycans and the genes responsible for their synthesis will be the topic of further exciting studies.

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Figure 1 Western blotting of *Acanthamoeba* extracts. The loaded amounts were optimized to give roughly equal Coomassie blue staining prior to blotting with either anti-horseradish peroxidase (A, anti-HRP) or anti-mannose 6-phosphate (B, anti-M6P). Molecular masses are indicated in kDa. Lanes 1-8 contain trophozoites of the following strains: (1) Neff, (2) PAT06, (3) 1BU, (4) ATCC 30234, (5) 72/2, (6) 11DS, (7) Pb30/40 (8) 4RE. For the anti-M6P blot, an extract of acid phosphatase-deficient mouse brain expressing high levels of M6P residues was used as positive control (Con) and shows the presence of prominent immunoreactive glycoproteins in the range between 40 and 60 kDa. Molecular weight markers are shown in kilodaltons (kDa).
Figure 2 The N-glycome of the *Acanthamoeba* 4RE strain. PNGase F released glycans were pyridylaminated and analysed by MALDI-TOF MS in the positive ion mode before (control) and after treatment with *Aspergillus* α1,2-mannosidase (α1,2-Man), endoglycosidase H (EndoH), jack bean α-mannosidase (JB) and a combination of jack bean α-mannosidase and endoglycosidase H (JB+Endo H). The glycans were detected primarily as [M+Na]$^+$ ions, whereas in the case of the sample treated with jack bean mannosidase [M+H]$^+$ ions dominated in the lower mass range.
Figure 3: Mass spectrometric analysis of a novel mannosylated core fucose epitope. The fragmentation pattern of the untreated sample of a glycan present in NP-HPLC fraction IV displaying an m/z [M+H]$^+$ of 1621 (A; composition Hex$_6$HexNAc$_2$Fuc$_1$) suggested the presence of a hexose attached to the core fucose (fragment ion at m/z 608). Based on the results of jack bean α-mannosidase (B; +JBM) and combined jack bean α-mannosidase and bovine α-fucosidase (C; +JMB+F) digestions (for MS data see the insets), after which in turn the m/z 608 and 446 fragments are no longer detected upon MS/MS of selected products, we postulate that a core α1,6-fucose is capped with an α-linked mannose. The data presented are from a glycan from the 4RE strain, but similar data (not shown) were obtained for one from the ATCC 30234 strain. The glycan structure and the core fragments are depicted according to the nomenclature of the Consortium for Functional Glycomics (circle, hexose; square, N-acetyllhexosamine; triangle, deoxyhexose).
Figure 4: MALDI-TOF mass spectrometric analysis of a phosphorylated glycan. A putatively phosphorylated glycan from the 4RE strain with an $m/z$ [M+H]$^+$ of 1717 (inset) was fragmented in positive-ion mode; the presence of fragments of $m/z$ 243 and 405 suggest the presence of phosphate on the $\alpha 1,3$-arm of the glycan. The cleavage putatively yielding the Hex$_3$HexNAc$_2$Phos-PA fragment is indicated with a dashed line.

Figure 5: Detection of methylation and core pentosylation of N-glycans. Of various MS/MS spectra of 2D-HPLC purified glycans isolated from the 4RE strain (RP-HPLC fractions derived from NP-HPLC fractions VI and IX), two were found to display hallmarks of methylation of a hexose close to the core and, in one case, pentosylation also close to the core; the Hex$_8$HexNAc$_2$Me glycan (A) is rather pure but the Hex$_9$HexNAc$_2$Pnt$_2$Me glycan (B) co-elutes with Man$_9$GlcNAc$_2$. In the MS/MS (LIFT) spectra (left-hand panels) the GlcNAc$_1$-PA fragment ($m/z$ 300) is set ‘off-scale’; the key intermediate fragments in the MS/MS spectra and possible overall structures are depicted.
Figure 6: Mass spectrometric analysis of the major Hex$_3$HexNAc$_2$Pnt$_1$ glycan from the 4RE strain. The major RP-HPLC fraction of the NP-HPLC fraction IX containing glycans of $m/z$ 1931 was subject to MALDI-TOF MS (A, B) and MS/MS (C, D) before and after overnight treatment with jack bean $\alpha$-mannosidase. MS/MS of the untreated $m/z$ 1931 glycan (C) and a mannosidase product of $m/z$ 1121 (D) indicate the presence of a fragment of $m/z$ 635 suggestive of pentosylation of the distal core GlcNAc. In the case of an authentic plant (bean) Man$_3$GlcNAc$_2$Xyl glycan (E) with the same molecular mass, the $m/z$ 635 fragment is absent from the MS/MS spectrum. The untreated glycan was also subject to perdeuteromethylation resulting in major products of $m/z$ 2510 and 2527 (F) as well as their sodiated forms. MS/MS of the perdeuteromethylated glycan (G) also corroborated the position of the pentose residue as probably being on the distal core GlcNAc.
Figure 7: Mass spectral analysis of large glycans from the 4RE strain modified with xylose, methyl and fucose. (A-F) The MALDI-TOF MS/MS spectra (range m/z 150-1850) of selected glycans identified in 2D-HPLC fractions (derived by RP-HPLC of NP-HPLC fractions XI, XII, XIII and XIV; see Supplementary Figures 4A and 5) are shown including two isomers of Hex$_{1}$HexNAc$_{2}$Pnt$_{2}$MeFuc which differ due to the absence (A) or presence (B) of a hexose ‘cap’ on the core fucose residue as indicated by a dominant m/z 446 fragment in the former and a dominant m/z 608 fragment in the latter; in panels C, E and F the m/z 608 fragment is set ‘off-scale’ in order to visualise the other fragments. (G-I) Selected regions of MS/MS (left) and MS (right) spectra of the Hex$_{12}$HexNAc$_{2}$Pnt$_{2}$Fuc glycan (see also panel E) and its jack bean mannosidase and combined mannosidase/fucosidase digestion products; alterations in the spectra are indicated as ‘-Hex’ and ‘-Fuc’. A possible, but not necessarily definitive, structure is shown.
Figure 8: Multiple-pentosylation of an N-glycan from the Pb30/40 strain. A glycan (HexHexNAc3Pnt4) purified by two-dimensional HPLC was subject to MS/MS before (A) and after (B) jack bean α-mannosidase digestion. Putative structures of the original glycan (m/z 2166) and its digestion product (m/z 1355) are shown.

Figure 9: Modifications of the reducing terminus in N-glycans isolated from pathogenic strains. The MS/MS spectra (range m/z 150-1150) of selected N-glycans from four strains originally isolated from clinical samples (72/2, 1BU, PAT06 and 11DS) show fragments of either m/z 446, 608 or 740, suggestive of core fucosylation with or without additional hexose and pentose residues.
**Table 1: Acanthamoeba strains used in this study.** Genotypes T4, T5, T6, T7 and T11 are based on the phylogeny of rRNA sequences, whereas the morphological groups are based on cyst morphologies. Pathogenic (P) and non-pathogenic (NP) strains are defined under consideration of whether the isolate was from a clinical source (with associated disease) or an environmental source (including, in the case of 4RE, a lens case of a subject with no keratitis).

| Strain | Genotype | GenBank (18S rRNA) | Morphological group | Pathogenicity | Identification | Origin |
|--------|----------|--------------------|---------------------|---------------|----------------|--------|
| 1BU    | T4       | AF260721           | II                  | P             | A. castellani  | cornea, keratitis patient |
| ATCC 30234 | T4     | AF239162           | II                  | NP            | A. castellani  | yeast culture, London     |
| Neff   | T4       | EF554328           | II                  | NP            | A. castellani  | soil               |
| PAT06  | T4       | EF429131           | II                  | P             | A. castellani  | cornea, keratitis patient |
| 72/2   | T5       | U94732             | III                 | P             | A. lenticulata | mouse brain, encephalitis |
| 11DS   | T6       | AF251939           | II                  | P             | A. hatchetti   | contact lens case and cornea, keratitis patient |
| Pb 30/40 | T7   | not available      | I                   | NP            | A. comandoni  | greenhouse          |
| 4RE    | T11      | AF251937           | II                  | NP            | A. hatchetti   | lens case, non-keratitis contact lens wearer |
Table 2: Most abundant N-glycans in eight *Acanthamoeba* strains: The data from initial glycomic screening are summarised to show \( m/z \) values and the predicted composition of the ten major N-glycans derived from PNGase F digests of glycopeptides from each of eight different *Acanthamoeba* strains; MS/MS was employed to predict the composition. As a means of semi-quantitative comparison between strains we calculated relative peak intensities of nine major glycans compared to the intensity of Hex5HexNAc2 \((m/z \ 1983)\), a glycan present in all strains (the relative intensity of this glycan is normalised to be 100). The glycans are abbreviated in the form of \( H_vN_wP_xF_yMe_z \), where \( H \) represents hexose \((\Delta m/z \ 162)\), \( N \) \( N\)-acetylhexosamine \((\Delta m/z \ 203)\), \( P \) pentose \((\Delta m/z \ 132)\), \( F \) fucose \((\Delta m/z \ 146)\) and \( Me \) a methyl group \((\Delta m/z \ 14)\). The genotypes (T4, T5, T6, T7 and T11) and the pathogenic/non-pathogenic (P or NP) status of each strain are also indicated.

| \( m/z \) \( [M+Na]^+ \) | Predicted composition | ATCC 30234 (T4; NP) | Neff (T4; NP) | 1BU (T4; P) | PAT06 (T4; P) | 72/2 (T5; P) | 11DS (T6; P) | Pb 30/40 (T7; NP) | 4RE (T11; NP) |
|----------------|-------------------|------------------|-------------|-------------|--------------|-------------|-------------|----------------|--------------|
| 1335 | H5N2 | 26 | 17 | 20 | 44 | 265 | 36 |
| 1497 | H6N2 | 30 | 30 | 26 | 20 | 74 |
| 1659 | H7N2 | 79 | 95 | 54 | 50 | 62 | 64 | 133 | 59 |
| 1761 | H6N2P2 | 21 | 21 | 21 | 21 | 61 |
| 1791 | H7N2P | 83 |
| 1821 | H8N2 | 66 | 86 | 49 | 47 | 65 | 83 | 111 | 72 |
| 1923 | H7N2P2 | 256 |
| 1953 | H8N2P | 56 | 68 | 87 | 24 | 81 | 90 | 430 |
| 1983 | H9N2 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 2055 | H7N2P3 | 283 |
| 2115 | H9N2P | 93 | 120 | 190 | 68 | 220 | 191 | 241 |
| 2145 | H10N2 | 71 |
| 2187 | H7N2P4 | 103 |
| 2247 | H9N2P2 | 16 |
| 2275 | H9N2P2Me2 | 57 |
| 2277 | H10N2P | 38 | 57 | 141 | 47 | 153 | 75 | 39 |
| 2333 | H7N2P5Me | 48 | 16 |
| 2361 | H7N2P2F3 | 33 |
| 2407 | H9N2P3Me2 | 38 |
| 2423 | H10N2P2Me | 65 |
| 2509 | H8N2P3F2 | 25 |
| 2523 | H8N2P2F3 | 27 |
| 2553 | H9N2P3FMe2 | 34 |
| 2641 | H8N2P5FMe | 14 |
| 2715 | H10N2P3FMe2 | 23 |
| 2731 | H11N2P2FMe | 62 |
| 2894 | H12N2P2FMe | 80 |
| 2964 | H9N2P5F2Me2 | 60 |
| 3096 | H9N2P6F2Me2 | 87 |
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