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Characterization of an α-L-fucosidase from the periodontal pathogen *Tannerella forsythia*

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Abbreviations: Amp, ampicillin; BHI, brain heart infusion medium; CBB, Coomassie Brilliant Blue G 250; DFJ, deoxyfuconojirimycin; Erm, erythromycin; TfFuc1, *T. forsythia* ATCC 43037 fucosidase-1 encoded by the *bfo_2737* gene, equally *Tffuc1*; FDH, fucose dehydrogenase; HPAEC, high-pH anion-exchange chromatography with pulsed amperometric detection; LC-ESI-MS, liquid chromatography-electrospray ionisation-mass spectrometry; rTfFuc-1, recombinant TfFuc1 enzyme; NAM, *N*-acetylmuramic acid; PBS, phosphate-buffered saline; pNP-fucose; 4-nitrophenyl-α-L-fucopyranoside; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; *T. forsythia*, *Tannerella forsythia* ATCC 43037; WT, wild-type bacterium

Abstract

The periodontal pathogen *Tannerella forsythia* expresses several glycosidases which are linked to specific growth requirements and are involved in the invasion of host tissues. α-L-fucosyl residues are exposed on various host glycoconjugates and, thus, the α-L-fucosidases predicted in the *T. forsythia* ATCC 43037 genome could potentially serve roles in host-pathogen interactions. We describe the molecular cloning and characterization of the putative fucosidase TfFuc1 (encoded by the *bfo_2737* = *Tffuc1* gene), previously reported to be present in an outer membrane preparation. In terms of sequence, this 51-kDa protein is a member of the glycosyl hydrolase family GH29. Using an artificial substrate, p-nitrophenyl-α-fucose (K_M 670 μM), the enzyme was determined to have a pH optimum of 9.0 and to be competitively inhibited by fucose and deoxyfuconojirimycin. TfFuc1 was shown here to be a unique α(1,2)-fucosidase that also possesses α(1,6) specificity on small unbranched substrates. It is active on mucin after sialidase-catalysed removal of terminal sialic acid residues and also removes fucose from blood group H. Following knock-out of the *Tffuc1* gene and analysing biofilm formation and cell invasion/adhesion of the mutant in comparison to the wild-type, it is most likely that the enzyme does not act extracellularly. Biochemically interesting as the first fucosidase in *T. forsythia* to be characterized, the biological role of TfFuc1 may well be in the metabolism of short oligosaccharides in the periplasm, thereby indirectly contributing to the virulence of this organism. TfFuc1 is the first glycosyl hydrolase in the GH29 family reported to be a specific α(1,2)-fucosidase.

Introduction

Tannerella forsythia is a Gram-negative anaerobic oral pathogen, a member of the so-called "red complex" of bacteria that cause a set of inflammatory diseases named periodontitis, affecting millions of people worldwide.[1-3] The effects on the periodontium include loss of the alveolar bone around the teeth, swelling and bleeding of the gum and, in more severe cases, loss of teeth. Periodontitis has also been linked to systemic inflammation and to an increased risk of stroke, heart attacks and atherosclerosis, amongst others.[4]

Like other bacteria residing in human hosts, *T. forsythia* has adapted to better suit its niche with cell surface glycosylation thought to be key to this adaptation.[5] As previously described, *T. forsythia* cells are completely covered by a unique surface (S-) layer formed by co-assembly of two different proteins both of which are highly *O*-glycosylated with an equally unique glycan.[6-8] Mutant strains lacking either the S-layer or glycan assembly and maturation genes, display phenotypes involving altered human cell attachment to host cells, biofilm formation, and disease progression.[9-11] In addition, the structure of the glycan partially imitates that of host glycoproteins, having a terminal sialic acid-like residue (precisely, a modified pseudaminic acid residue) and a terminal fucose, with the latter shown to be present in substoichiometric amounts and linked to a methylated galactose in an unknown glycosidic linkage.[8] The glycobiology of this pathogen, including its repertoire of glycosidases, seems to be key to its physiology and, potentially, its pathogenicity.[5, 12]

Recent evidence suggests that for several periodontal pathogens, but particularly for the "red complex" organism *T. forsythia*, sialic acid-containing host molecules play an important role *in vivo*.[13] Two different sialidases have been found in *T. forsythia*, SiaHI and NanH. In the case of SiaHI, its function is unclear. It is not a canonical sialidase (*i.e.,* not in the GH35 family), a *siaH1* mutant has no discernible phenotype, and experiments point to it being a periplasmic protein without any role in extracellular interactions.[14] These same studies

also indicated that mutants lacking the main *T. forsythia* sialidase NanH had hindered attachment and invasion of human oral epithelial cells. The enzyme was also seen to play an important role in biofilm growth on surfaces coated with salivary glycoproteins.[15] The *nanH* gene is located in a large cluster that contains all the genes required for sialic acid catabolism, which indicates that the cleaved sialic acid can additionally be taken up and utilized.[12, 13] This gene cluster also contains a β-hexosaminidase that may cleave sub-terminal residues after sialidase action and may also play a role *in vivo*.[15] Additionally, in a separate study, transcriptome analysis of the oral microbiome also showed up-regulation of the sialidase *nanH* mRNA in dental plaque.[16]

α-L-fucosyl residues are, like sialic acid, frequently located at a terminal position on various host glycoconjugates including blood groups, milk oligosaccharides, gastric and submaxillary mucin, and serum glycoproteins.[17, 18] Therefore, fucosidases in *T. forsythia* could potentially play similar roles to sialidases.[19] It has generally been shown that terminal fucose residues play important roles in mammalian cell-cell communication and also in their interaction with pathogenic bacteria; for instance, *Campylobacter jejuni* and *Helicobacter pylori* are known to bind certain fucosylated blood groups (*e.g.,* O-antigen) in order to mediate infection.[19-21] In addition, the ability to utilize available fucose provides many bacteria with a nutritional advantage and contributes to survival in a highly competitive ecosystem, such as the human body.[22, 23]

Characterization of α-L-fucosidases in *T. forsythia* could aid in the elucidation of the structure-function relationship of fucosylated host and bacterial surfaces in the virulence of oral pathogens. The genome of *T. forsythia* encodes three putative α-L-fucosidases, BFO_2737 and BFO_1182, both classified in the CAZy (Carbohydrate Active enZymes; http://www.cazy.org/) glycosyl hydrolase family GH29, and BFO_3101, classified in family GH95. While all three enzymes possess a glycosyl hydrolase domain and are classified by CAZy according to their mechanism of action, BFO_1182 and BFO_3101 are not strictly

annotated as α-L-fucosidases but as a F5/8 type C domain protein and a putative lipoprotein, respectively.

Here, we describe the molecular cloning and characterization of BFO_2737, which we named TfFuc1. This protein has previously been reported to form part of an outer membrane preparation of *T. forsythia*[24] and, thus, was a good candidate to be involved in host-pathogen interactions. TfFuc1 is a 446-amino acid protein with a theoretical pI and molecular mass of 6.9 and 50.8 kDa, respectively. It is the first fucosidase in this organism to be characterized to date. The enzyme was shown here to be an α(1,2)-fucosidase and also possesses an α(1,6) specificity on small unbranched substrates. It is a predicted periplasmic protein, possibly playing a role in the breakdown of small oligosaccharides. It is, to the best of our knowledge, the first glycosyl hydrolase in its family (GH29) reported to be a specific α(1,2)-fucosidase.

Results

Enzymatic characterization of rTfFuc1. The *Tffuc1* gene was cloned into pET22-b(+) vector and expressed in *E. coli* as a C-terminally His_6-tagged protein, which enabled purification via nickel affinity chromatography (**Fig. 1**). The enzymatic activity was then tested using the standard colorimetric α-fucosidase substrate 4-nitrophenyl-α-L-fucopyranoside (pNP-fucose) at 22°C in a range of different pH values and in the presence of $MgCl_2$, KCl and NaCl, in order to establish its pH optimum and cation dependence, respectively. By stopping the reaction with the addition of an alkaline buffer at pH 11.4, it was ensured that all wells where at the same pH for consequent absorbance readings. The activity of the enzyme was seen to start to plateau at the neutral to alkaline pH range and was considered most active at pH 9.0, assayed in glycine buffer, and not at pH 9.25 where the activity suddenly peaks and then rapidly decreases thereafter. The activity remained largely unaffected by the presence of cations at the two concentrations tested (results not shown). The

K_M and V_{max} catalytic constants at 22°C, calculated from the activity of the enzyme at different pNP-fucose concentrations, were 670 µM and 20.4 µmol/min (U) per mg of protein, respectively (**Table 1**). The determined catalytic constants for rTffuc1 are in the range of those reported for other fucosidases/ glycosylhydrolases when tested on their corresponding pNP-substrates.[25-27]

Substrate linkage specificity of rTfFuc1. To determine the enzyme linkage specificity, rTfFuc1 was incubated with a set of different fucosylated substrates of defined structure representing a range of fucose linkages available on host glycoproteins and on oral surfaces (**Fig. 3**). The reaction products obtained after overnight incubation were analysed using high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC) where the release of fucose was confirmed by comparison with the retention time of the standard monosaccharide and of a substrate standard after overnight incubation at 37°C. The enzyme was seen to be active on both α(1,2) fucose containing substrates, 2-fucosyllactose and H-trisaccharide, and on the α(1,6) fucose disaccharide α-L-Fuc-(1,6)-β-D-GlcNAc, although this latter reaction did not reach completion, indicating weak specificity for this linkage. The α(1,3) and α(1,4) linkages were not cleaved on 3-fucosyllactose and the Lewis A trisaccharide, respectively. The enzyme was also inactive on the substrate α-L-Fuc-(1,4)-β-D-Gal, added as a second disaccharide control to prove that the α(1,6) activity was not due to differences in substrate length (**Fig. 4**).

In order to obtain accurate activity values on the cleaved substrates, the K-FUCOSE kit from Megazyme was used, coupled to the enzymatic reaction with rTfFuc1. First, FDH, which also has an alkaline pH optimum, and $NADP^+$ were added to the substrate solution reaction mixture in order to convert any free fucose already present in the sample to L-fucono-1,5-lactone by the reduction of $NADP^+$ to NADPH ($\varepsilon_{340} = 6.022\ mM^{-1}cm^{-1}$). rTfFuc1 was then added to the mixture and the reaction was monitored by following the increase in Abs_{340}. The

activity was calculated from where the formation of NADPH was linear over time. The enzyme was most active on 2-fucosyllactose and H-trisaccharide with specific activities of 0.8 U/mg and 0.6 U/mg, respectively. The activity on the α(1,6) disaccharide was significantly lower at 0.35 U/mg (**Table 2**).

The activity of the enzyme on the various substrates could be calculated approximately (as some loss of material occurred during sample preparation) from the HPLC experiments after 1-h incubation periods (results not shown) and was found to be markedly lower than that observed with the K-FUCOSE kit, indicating that free fucose, which is consumed in the latter, could be inhibiting the enzymatic activity significantly. In order to determine the extent of such an effect, measurement of K_M and V_{max} values were repeated with pNP-fucose in the presence of either 0.25 mM L-fucose or 0.1 μM deoxyfuconojirimycin (DFJ), which is a strong fucosidase inhibitor.[28] The enzyme was competitively inhibited by both fucose and DFJ as the V_{max} remained largely unaffected but the K_M value increased from 0.67 mM to 16.5 mM and 28.3 mM, respectively (**Table 1**).

Further, the ability of the enzyme to cleave fucose residues off more complex natural glycans and those on branched sugar residues was assayed (compare with **Fig. 3**). As expected, the enzyme was unable to cleave the α(1,3) fucose linkage on GalFGalF-pep, included in the assays as a trace amount of activity could be observed when using 3-fucosyllactose, as measured by the K-FUCOSE kit. The enzyme was also not able to cleave the core α-1,6 fucose linkage on $GnGnF^6$-pep nor the branched α(1,2)-linked fucose on the A antigen. The non-branched α(1,2) fucose linkage present on the Eastern oyster substrate, however, was cleaved off the substrate GalF, seen by the loss of a fucose residue in the MS spectra of the substrate. The major m/z 1703 glycan ($[M+H]^+$) was approximately 50% digested to a defucosylated species of m/z 1557 after overnight incubation with the enzyme (**Fig. 5**). The enzyme is, therefore, able to cleave off fucose residues which are α(1,2) linked on more complex glycans only when in a terminal unbranched position and is unable to cleave

core α(1,6) fucose. This data supports that the enzyme acts as an α(1,2) fucosidase.

rTfFuc1 activity on bovine submaxillary mucin. rTfFuc1 was incubated with mucin from bovine submaxillary glands and the release of fucose was measured with the K-FUCOSE kit. Incubation was performed also in combination with rNanH from *T. forsythia* and activities were calculated from the slope of ΔAbs_{340} where it was linear over time. During the assayed incubation period of 10 min, no activity could be detected when rTfFuc1 was incubated alone with mucin. The ΔAbs_{340} lead to an irregular data set with a very low r^2 value. However, fucose release was detectable when the incubation was performed in conjunction with the rNanH sialidase, presenting a slow but steady increase in the Abs_{340}. The activity was calculated over a period of 300 s where the data points fitted a linear regression with an r^2 of 0.98 (**Fig. 6**). The experiment was repeated several times and yielded an activity of 24 ±4 mU/mg. rTfFuc1 might cleave fucose off mucin over longer periods of time, but the data shows a significantly higher activity when sialic acid residues are first removed from mucin, indicating that the fucosidase TfFuc1 could work downstream from the sialidase in *T. forsythia* and presumably cooperate with other glycosidases in the degradation of complex glycans.

Cellular localization of TfFuc1. TfFuc1 was previously reported to be present in the outer membrane fraction of *T. forsythia*.[24] In an effort to investigate its presence on the surface of *T. forsythia* cells, TfFuc1-specific polyclonal antiserum was raised against the recombinant enzyme in mice and used for Western immunoblotting of cellular fractions separated by SDS-PAGE. Protein visualization by CBB staining showed good separation between the fractions, as the S-layer bands were very prominent in the outer membrane fraction but not in the inner-membrane and non-membrane associated fractions. Western immunoblotting showed that all the detectable TfFuc1 fucosidase was found in the non-membrane associated fraction

comprising both the cytoplasmic and periplasmic content (**Fig. 7**), arguing against surface localization of the TfFuc1 enzyme.

Discussion

Colonization of the periodontal pocket by the pathogenic late colonizer *T. forsythia* depends largely on pre-existing bacteria that have already tipped the oral balance away from health and towards disease.[29] Factors such as a pH shift from neutral to alkaline and slight raises in the temperature due to the host inflammatory response could be contributing factors favouring the process.[30] In a situation of oral disease, the number of different bacteria living in the gingival crevice decreases markedly due to putative pathogenic bacteria being more competitive in such an environment.[31] It is in these conditions that *T. forsythia* seems to thrive and becomes one of the key players in severe cases of periodontitis.

The NanH sialidase in *T. forsythia* has been well established to play important roles in adherence to sialylated glycoprotein-coated surfaces and epithelial cells in addition to triggering biofilm growth and being up-regulated in dental plaque.[14-16] As the other important terminal sugar on host glycoproteins is fucose, here, we performed an initial characterization of a putative α-L-fucosidase encoded in the *T. forsythia* genome, product of the gene *Tffuc1*, previously reported to be part of the outer membrane proteome.[24]

By producing the enzyme recombinantly in *E. coli* we were able to show that the enzyme is active across a broad pH range from 7.0-9.0, having an unusually high pH optimum of 9.0. It presents a unique α(1,2)-linkage specificity on terminal non-branched fucose residues, being also active on small non-branched α(1,6) fucosylated substrates. Whilst both these linkages are cleaved at a considerable rate in the case of small linear substrates, the α(1,6) specificity is not detected on core fucoses on more complex glycopeptides. The α(1,2) linkage specificity was apparent on both small linear substrates, such as 2-fucosyllactose and

H-trisaccharide, and on more complex glycans only when fucose occupied a terminal position, but not on a branched substrate where the fucose residue is linked to a fully substituted sugar. The enzyme seems to be, to the best of our knowledge, the first fucosidase in its GH family (GH29) to have a specific α(1,2) activity. The broad, high pH activity profile of this fucosidase ties in with its physiological niche which is known to have a pH that rises as periodontal disease progresses.[30] The possession of such enzymes with higher activities in alkaline surroundings could contribute to competiveness and virulence of *T. forsythia* in a diseased environment.

During the course of this study, it became clear that one of the issues possibly underlining our observations was the enzyme's cellular localization. Even though TfFuc1 was found previously to be present in the outer membrane proteome of the pathogen,[24] localization of the enzyme on the surface of *T. forsythia* cells by fluorescent immunolabelling was not successful (data not shown; see Experimental Procedures in the Supplementary Information) and cell fractionation also showed the detectable protein to be found in the non-membrane associated fraction (**Fig. 7**). This would be in agreement with bioinformatics interrogation indicating that, while it has a Sec-dependent secretion signal (as predicted by SignalP 4.1 Server), it is not predicted to reside in the outer membrane or be secreted, nor does it contain a T9SS C-terminal secretion signal.[32] The difference in location of TfFuc1 between the present study and the outer membrane proteomics experiment[24] can be reconciled when considering that in the latter study, cross-contamination of individual proteins between cellular fractions was not investigated.

Incubation of the recombinant fucosidase with bovine submaxillary mucin showed no detectable release of fucose over an incubation period of 10 min. Activity on this complex substrate could only be detected when the incubation was performed in combination with the recombinant NanH sialidase from *T. forsythia*. It is, therefore, conceivable that TfFuc1 could play an accompanying role to the sialidase in the interaction between *T. forsythia* and host

glycoproteins, but given its periplasmic location, this could merely reflect the need for removal of terminal sialic acid residues for the enzyme to work, either indicating that it most likely acts on internalised fucosyl substrates after sialic acid has already been removed by the action of sialidases or, less likely, that it acts in concert with sialidases externally.

This notion that the *T. forsythia* fucosidase TfFuc1 plays an internal role was corroborated when we tested the effect of the Δ*Tffuc1* mutation on the ability of *T. forsythia* to interact with and invade human oral epithelial cells using an antibiotic protection assay on the oral epithelial cell line H357.[33] We found no significant differences in the ability of the Δ*Tffuc1* to invade these human cells as compared to the WT strain (**Fig. S1)**, indicating that TfFuc1 has no effect on epithelial cell-invasion under the conditions tested. In addition, the mutant did not show hindered biofilm formation when cultured on bovine submaxillary mucin, contrary to the *T. forsythia NanH* sialidase mutant,[15] but showed a slightly increased biofilm formation (**Fig. S2**).

Our data supports the idea of a periplasmic fucosidase involved in the final breakdown of small substrates that have been internalized, possibly owing to the action of exoglycosidases and endoglycosidases which break-down larger glycans on the outside of the cell.[34, 35] TfFuc1 would possibly then be able to exert its full potential freeing both α(1,2) and α(1,6) fucoses on small linear substrates. These findings are also in agreement with the hypothesis that the fucosidase acts downstream of the sialidases, which have been shown to act on whole glycoproteins on bacterial and host surfaces.[14, 15, 36] The sialic acid would, therefore, already be missing as smaller parts of the glycan are transported inside the cell.

T. forsythia has no straightforwardly identifiable fucose catabolism locus in its genome, nor does it have the bifunctional L-fucokinase/GDP-fucose pyrophosphorylase required normally for *Bacteroidetes* to recycle the fucose into its glycans.[23] In an effort to see the effect on the latter scenario, the fucose containing S-layer glycan from both the WT and the Δ*Tffuc1* strains were compared by LC-ESI-MS with no obvious change under the growth

conditions used (Z.A. Megson, L. Neumann, F. Altmann, C. Schäffer, unpublished data). However, the microheterogeneity of the S-layer glycan regarding the terminal fucose residue complicates interpretation of MS data. Therefore, it remains unclear whether the released fucose in the periplasm can be used as a nutrient source or is recycled by the bacteria into its glycosylation pathway, and, thus, is subject of further studies.

Overall, our data suggest that TfFuc1 is a unique α-L-(1,2)-fucosidase which could potentially contribute to fucose utilization in *T. forsythia*. In order to better elucidate this role and rule out any redundancy in the system, two further annotated fucosidases in *T. forsythia*, BFO_1182 and BFO_3101, together with the annotated fucose permease, BFO_0307, are now being investigated to elucidate the role of fucose in the physiology of *T. forsythia*.

Experimental Procedures

Bacterial strains, medium and culture conditions. *T. forsythia* wild-type (WT) strain ATCC 43037 (American Type Culture Collection) and the knock-out mutant Δ*Tffuc1* were grown anaerobically at 37°C for 4-7 d in brain heart infusion (BHI) broth or 0.8% (w/v) BHI agar, supplemented with *N*-acetylmuramic acid (NAM), horse serum and gentamycin as described previously.[32] *Escherichia coli* DH5α and BL21 (DE3) (Invitrogen) were cultivated in selective Luria Bertani (LB) medium (agar and broth) supplemented with 100 µg/ml ampicillin (Amp). All strains and plasmids used in the course of this study are summarised in **Table 3**.

Molecular methods. All enzymes were purchased from Fermentas. Genomic DNA of *T. forsythia* WT strain ATCC 43037 was isolated from 2 ml of bacterial suspension as described previously and used as the DNA template in all PCRs, unless otherwise specified.[37] The

GeneJET™ Gel Extraction Kit (Fermentas) was used to purify DNA fragments from agarose gels and to purify digested plasmids and oligonucleotides. Plasmid DNA from transformed cells was isolated with the GeneJET™ Plasmid Miniprep kit (Fermentas). Agarose gel electrophoresis was performed as described elsewhere.[38] Primers for PCR and DNA sequencing were purchased from Invitrogen (**Table 4**). PCR was performed using the Phusion®High-Fidelity DNA Polymerase (Fermentas) and a My CyclerTM (Bio-Rad) thermal cycler. Transformation of chemically competent *E. coli* DH5α and BL21 (DE) cells was performed according to the manufacturer's protocol (Invitrogen). *E. coli* transformants were screened by PCR using RedTaq ReadyMix PCR mix (Sigma-Aldrich) and recombinant clones were analyzed by restriction mapping. Expression vector and knock-out cassette were sequenced (Microsynth) prior to transformation.

Recombinant production of His_6-tagged TfFuc1. The *Tffuc1* gene was amplified from the chromosome of *T. forsythia* ATCC 43037 with a fused C-terminal His_6-tag by PCR using primer pair 1/2 (**Table 4**). The His_6-tagged amplification product was digested using restriction enzymes NdeI/XhoI and cloned into NdeI/XhoI-linearized pET22-b (Novagen). The corresponding plasmid was transformed into *E. coli* BL21 (DE3) cells for protein expression. Freshly transformed cells were grown in two 400-ml Erlenmeyer flasks to an OD_{600} of 0.4-0.5 in the presence of 100 µg/ml of Amp at which point protein expression was induced with a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and cultures were shaken (200 rpm) overnight at 18°C. Cells were harvested by centrifugation (6,500 *g*, 20 min, 4°C).

Collected bacterial cells were lysed by sonication in buffer A (50 mM phosphate buffer pH 8, 0.3 M NaCl) containing 5 mM imidazole and cleared lysates after ultracentrifugation at 150,000 *g* for 30 min at 4°C were incubated with 1 ml of Ni-NTA beads (Qiagen) for 1 h at 4°C, shaking slightly. The beads were placed in a chromatography column

and the His_6-tagged protein was purified using an imidazole gradient in buffer A; 25 mM imidazole (10 ml), 50 mM imidazole (10 ml), followed by five elution steps with 500 µl of 250 mM imidazole in buffer A. Eluted fractions containing the purified recombinant protein, rTfFuc1, as determined by SDS-PAGE analysis, were pooled and dialysed overnight at 4°C against 3 l of 10 mM phosphate buffer, pH 8.0. The volume was then reduced 5-fold using a concentration centrifuge yielding a protein concentration of 0.35 mg/ml (as determined by Nanodrop) in 50 mM phosphate buffer.

Construction of a *T. forsythia* Δ*Tffuc1* knock-out strain. Disruption of the *Tffuc1* gene in *T. forsythia* was performed by gene knockout, as described previously.[32] The *Tffuc1* gene is not part of an operon, thus, downstream effects due to the chosen mutation strategy are not expected to occur. Briefly, the flanking genomic regions (1,000 bp) up-stream and down-stream of *Tffuc1* were amplified using primer pairs 3/4 and 5/6, respectively (**Table 4**). The two resulting fragments were joined with the erythromycin resistance gene *ermF-ermAM* (amplified using primer pair 7/8) by overlap extension PCR and sub-cloned into the blunt-end cloning vector pJET1.2 (Thermo Scientific), resulting in pJET1.2/*Tffuc1*_ko. Approximately 5 µg of the knockout cassette was transferred by electroporation into 100 µl of competent *T. forsythia* cells. Cells were regenerated in BHI medium for 24 h before plating on BHI agar plates containing erythromycin (10 µg/ml) as a selection marker. Single colonies were picked and used for inoculation of liquid BHI medium. Genomic DNA of the new Δ*Tffuc1* mutants were isolated as mentioned above and the absence of the *Tffuc1* gene and the correct integration of the erythromycin resistance gene (upstream and downstream) was evaluated by PCR using primer pairs 1/2, 9/10, and 11/12, respectively (**Table 4**). Absence of the enzyme in the Δ*Tffuc1* strain was also confirmed by Western immunoblotting of the total cell extract separated by SDS-PAGE using TfFuc1-specific polyclonal antiserum (**Fig. 1**).

General and analytical methods. SDS-PAGE was carried out according to a standard protocol using a Protean II electrophoresis apparatus (Bio-Rad).[39] Protein bands were visualized with Coomassie Brilliant Blue G 250 (CBB) staining reagent. For Western immunoblotting of proteins onto a nitrocellulose membrane (Peqlab), a Mini Trans-Blot Cell (Bio-Rad) was used. Detection of the His_6-tag fused to rTfFuc1 and detection of TfFuc1 was done with the Li-Cor Odyssey Infrared Imaging System using an anti-His_6 mouse antibody (Roche) or TfFuc1-specific polyclonal antiserum raised in mice (EF-BIO), respectively, both in combination with goat anti-mouse IgGIR Dye 800CW conjugate (Li-Cor).

Enzymatic characterization of rTfFuc1. Enzymatic characterization of rTfFuc1 was performed essentially as described elsewhere.[40] 0.17 µM of purified, recombinant enzyme was incubated with 5 mM of the colorimetric substrate 4-nitrophenyl-α-L-fucopyranoside (pNP-fucose) (Sigma) at a range of different pH values (3-10.25) and cation concentrations in 96-well plates at 22°C in a total volume of 40 µl. The enzymatic reaction was stopped after 3 min by addition of 260 µl of phosphate buffer, pH 11.4. Citrate/phosphate buffer (0.1 M) was used to assay the pH range from 3-8,[41] 50 mM glycine buffer was used for a pH range from 8-10. The effect of $MgCl_2$ (5 mM, 10 mM), KCl (5 mM, 10 mM) and NaCl (50 mM, 150 mM) on the enzyme's activity was assayed in the same way in 50 mM glycine buffer, pH 9.

A 4-nitrophenol standard curve was made by measuring the absorbance at 405 nm (Abs_{405}) of 0, 4, 8, 12, 16, 20 and 24 nmol of 4-nitrophenol per well in 300 µl of phosphate buffer, pH 11.4. The K_M and V_{max} catalytic constants were calculated at pH 9.0 in 50 mM glycine buffer at 22°C in the presence of 0.01 to 50 mM pNP-fucose. The inhibitory effect of fucose and deoxyfuconojirimycin (DFJ) on the K_M and V_{max} of the enzyme were assayed in the same way in the presence of 0.25 mM fucose and 0.1 µM DFJ, respectively.[28] Readings were performed using an Infinite 200 plate reader (TECAN) and catalytic constants

were calculated with the Sigma Plot 12, Systat Software.

Substrate specificity of rTfFuc1. For the determination of enzyme linkage specificity, a set of commercially available fucosylated substrates (2-fucosyllactose and 3-fucosyllactose from Dextra laboratories; H-Trisaccharide, Lewis A trisaccharide, Fuc-α-(1,4)-Gal and Fuc-α-(1,6)-GlcNAc, all from Carbosynth) (**Fig. 3**) were incubated with the enzyme and reaction mixtures were analysed by HPAEC using an ICS3000 chromatographic system (Dionex, Thermo Fisher) on a CarboPac PA-1 column. Incubations were made overnight at 37°C in a total volume of 100 µl by mixing 0.34 µM of enzyme with 0.5 mM of substrate in 50 mM glycine buffer, pH 9.0. In order to minimize the effect of the buffer, the reaction volume was then diluted with 400 µl of Milli-Q water and the enzyme was removed using an Amicon 3 kDa cut-off spin column (Millipore). Twenty-five microliters of this flow through was then applied to the CarboPacPA-1 column using full-loop injection.

In order to obtain reliable activity values, the K-FUCOSE kit (Megazyme) was adapted to suit requirements. In a total volume of 250 µl of 50 mM glycine buffer at pH 9.0, the substrates 2-fucosyllactose, H-trisaccharide and Fuc-α(1,6)-GlcNAc, were incubated separately at a concentration of 0.5 mM with 1.83 µl of fucose dehydrogenase (FDH) and 9.15 µl of $NADP^+$ (both as supplied) in a cuvette at 37°C for 10 min. When the reaction had reached a constant absorbance at 340 nm (Abs_{340}), rTfFuc1 was added to the mixture at a concentration of 0.34 µM and the formation of NADPH was followed by continuous measurement of the increase in Abs_{340}. The activity of the enzyme on each substrate was calculated according to the supplier's specifications from ΔAbs_{340}/min where the formation of NADPH was linear over time. The experiment was repeated with different enzyme dilutions (1:10; 1:100) to prove the reliability of the method.

The ability of the enzyme to cleave fucose residues off more complex natural glycans and those on branched sugar residues was assayed on the substrates A antigen tetraose type 5

(Carbosynth), GnGnF^6-peptide, GalFGalF, and an *N*-glycan derived from *Crassostrea virginica* (Eastern oyster) haemocyte treated with chicken liver α-*N*-acetylgalactosaminidase and bovine β-galactosidase (both from Sigma) to reveal the underlying H epitope presenting a terminal α(1,2) fucose on an unsubstituted β(1,3)-galactose (*i.e.,* histo blood group antigen H), referred to here as GalF^{42} (**Fig. 3**). Activity on the A antigen substrate was assayed using the K-FUCOSE kit as described above. For all other substrates, incubations were performed overnight at 37°C and analysed by MALDI-TOF MS using an Autoflex Speed instrument (Bruker) in positive ion mode with 6-aza-2-thiothymine (ATT) as matrix. 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 then manually interpreted.

rTfFuc1 activity on mucin from bovine submaxillary glands. rTfFuc1 was incubated with bovine submaxillary mucin (Sigma) in combination with the His-tagged recombinant NanH sialidase (rNanH) from *T. forsythia*.[15] Incubations were performed at 37°C in 50 mM glycine buffer, pH 9.0, at a final concentration of 0.1 µM of either enzyme, 0.2 mg/ml of mucin and $NADP^+$ and FDH as described above, in a total volume of 250 µl. rTfFuc1 (and rNanH) was added to the mixture after an initial 5-min incubation period. The release of fucose (ΔAbs_{340}) was followed over 10 min and calculated according to the supplier's specifications from ΔAbs_{340}/min where the formation of NADPH was linear over time.

Presence of TfFuc1 in *T. forsythia* membrane, outer membrane and non-membrane preparations. Cells were harvested by centrifugation from a 4-day-old 100-ml *T. forsythia* culture. Separation of cellular fractions was performed as described previously.[24] Briefly, cells were washed once in Tris (2-amino-2-hydroxymethyl-propane-1,3-diol)-buffer, pH 7.5, sonicated, and cell debris were removed by centrifugation. The collected supernatant was

ultracentrifuged (100,000 *g*, 4°C, 40 min) to separate the whole membrane fraction (pellet) from the membrane non-associated fraction (cytoplasm and periplasm, supernatant). The pellet was resuspended in 2% (w/v) N-lauroylsarcosine (Sigma) in Tris buffer and mixed. After incubation (2 h, 25°C), the outer membrane fraction (OM) was collected by centrifugation (100,000 *g*, 4°C, 40 min) and the pellet was resuspended in Tris buffer. The protein content was determined in each fraction by the Bradford method (Bio-Rad).[43] A total of 20 µg of protein from the OM and non-membrane associated fractions and 400 µg of the membrane fraction was loaded onto an SDS-PAGE gel and ran as described above. The presence of TfFuc1 in each fraction was determined by Western immunoblotting.

Microtiter assays of Hoechst-stained biofilms. *T. forsythia* WT and Δ*Tffuc1* strains were compared in respect to the biofilm formation[44] on mucin-coated polystyrene microtiter plates in dependency of the strength of the BHI liquid medium. Biofilm was stained with Hoechst 33258 Fluorescent Stain (Thermo Scientific). Details are described in the Supplementary Information.

Attachment and invasion assays. For both attachment and invasion assays, *T. forsythia* WT and Δ*Tffuc1* strains were incubated with the oral epithelial cell line H357 (CCL17; American Type Culture Collection) at a multiplicity of infection of 1:100, as described previously.[45] Details are described in the Supplementary Information

Disclosure and Potential Conflict of Interest

No potential conflicts of interests were disclosed.

Acknowledgements

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Figure legends

Figure 1. SDS PAGE (**A.**) and Western immunoblot (**B.**) of total cell extracts from *T. forsythia* WT (lane 2) and Δ*Tffuc1* strains (lane 3) and of the His_6-tagged rTfFuc1 as purified from *E. coli* (lane 4), used for activity studies and to raise a polyclonal anti-TfFuc1 antiserum. Western immunoblotting using the anti-TfFuc1 antiserum recognized the protein (~51 kDa) specifically in the WT strain (lane 2) and indicated absence of the protein in the Δ*Tffuc1* strain (lane 3), proving that the enzyme was effectively knocked-out. In the preparation of rTfFuc 1 (B., lane 4), the polyclonal antiserum recognizes also minor contaminating *E. coli* proteins not visible on the SDS-PAGE gel (A., lane 4). Mm; PageRuler Plus prestained protein ladder (Thermo Scientific).

Figure 2. pH profile of rTfFuc1 using 4-nitrophenyl-α-L-fucopyranoside (pNP-fucose) as a substrate. Activity was measured as the increase in Abs_{405} due to the released 4-nitrophenol product. Citrate/phosphate buffer (0.1 M) was used to assay the pH range from 3-8, 50 mM glycine buffer was used for the pH range from 8-10.25.

Figure 3. Fucosylated substrates used in this study. The structures are depicted according to the symbolic nomenclature of the Consortium for Functional Glycomics (http://www.functionalglycomics.org/static/consortium/Nomenclature.shtml).

Figure 4. rTfFuc1 activity on standard fucosylated substrates after overnight incubation as determined by HPAEC. Blue lines represent samples which were incubated in absence of rTfFuc1 (substrate standard) and red lines represent samples incubated in the presence of rTfFuc1. Cleavage of the substrates was determined by the appearance of a fucose peak, as determined by the retention time of the standard monosaccharide.

Figure 5. Cleavage of natural α(1,2) fucosylated glycans by rTfFuc1**.** Cleavage of fucose from a large *N*-glycan substrate was monitored by MALDI-TOF MS spectra after overnight incubation; the conversion of the *m/z* 1703 glycan (GalF) to one of *m/z* 1557 (*Δm/z* 146) is indicative of the loss of fucose. The structures of the substrate and product are depicted according to the symbolic nomenclature of the Consortium for Functional Glycomics.

Figure 6. rTfFuc1 was incubated with mucin from bovine submaxillary glands and the release of fucose was measured with the K-FUCOSE kit. When incubations were performed in conjunction with the rNanH sialidase, a slow steady increase in the Abs_{340} was observed. The activity was calculated over a period of 300 s where the data points fitted a linear regression with an r^2 of 0.98. No activity could be detected, when rTfFuc1 was incubated alone with the mucin. The ΔAbs_{340} lead to an irregular data set with a very low r^2 value of 0.4.

Figure 7. Presence of TfFuc1 in cell fractions of *T. forsythia* WT. **A.** SDS-PAGE analysis of the outer membrane fraction (OM) (1), membrane fraction (2) and non-membrane associated fraction (3) showed good separation between the fractions, as the S-layer bands were very prominent in the OM but not in the membrane and non-membrane associated fractions. Protein loaded was 20 µg of the OM and non-membrane associated fractions and 400 µg of the membrane fraction. Protein visualization was by CBB. **B.** Western immunoblot using anti-TfFuc1 antiserum showed the TfFuc1 fucosidase in the non-membrane associated fraction comprising both the cytoplasmic and periplasmic content. Mm; PageRuler Plus prestained protein ladder (Thermo Scientific).

Table 1. rfFuc1 activity on 4-nitrophenyl-α-L-fucopyranoside (pNP-fucose). Catalytic constants K_M and V_{max} and the inhibitory effect of DFJ and L-fucose were measured using the colorimetric substrate pNP-fucose within a concentration range from 0.01 to 50 mM at 22°C in glycine buffer at pH 9.0

Substrate*	Inhibitor	K_M (mM)	V_{max} (U/mg)**
pNp-fucose	None	0.67 (±0.2)	20.4 (±0.8)
pNp-fucose	0.1 µM DFJ***	28.3 (±3.7)	28.1 (±2.4)
pNp-fucose	0.25 mM Fucose	16.5 (±4.7)	28.6 (±4.4)

*4-nitrophenyl-α-L-fucopyranoside

**µmol/min/mg of enzyme

***deoxyfuconojirimycin

Table 2. rTfFuc1 activity on standard fucosylated substrates. Cleavage was determined by HPAEC after overnight incubations with rTfFuc1 (see **Fig. 4**, with the exception of A antigen tetraose) and specific activities were calculated using the K-FUCOSE kit

Substrate	Structure	Fucose linkage	Enzyme cleaves	Activity (U/mg)*
2-Fucosyllactose	α-L-Fuc-1,2-β-D-Gal-1,4-D-Glc	α(1,2)	Yes	0.8
3-Fucosyllactose	β-D-Gal-1,4(α-L-Fuc-1,3)-D-Glc	α(1,3)	No	-
H-trisaccharide	α-L-Fuc-1,2-β-D-Gal-1,3-β-D-GlcNAc	α(1,2)	Yes	0.6
Lewis A trisaccharide	β-D-Gal-1,3(α-L-Fuc-1,4)-β-D-GlcNAc	α(1,4)	No	-
Fuc(1,6)GlcNAc	α-L-Fuc-1,6-β-D-GlcNAc	α(1,6)	Yes	0.35
Fuc(1,4)Gal	α-L-Fuc-1,4-β-D-Gal	α(1,4)	No	-
A antigen tetraose	β-GalNAc-1,3(α-L-Fuc-1,2)-β-D-Gal-1,4-D-Glc	α(1,2)	No	-

*μmol/min/mg of enzyme

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant characteristic(s)	Source
Escherichia coli DH5α	F$^-$ Φ80d*lacZ* M15 (*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (rK$^-$mK$^-$) *phoA supE44 thi-1 gyrA96 relA1*$^-$	Invitrogen
Escherichia coli BL21 (DE)	*F*$^-$*, ompT, hsdS* (*rB*$^-$*mB*$^-$), *gal, dcm* (DE3)	Invitrogen
Tannerella forsythia ATCC 43037	Wild-type isolate	American Type Culture Collection, USA
T. forsythia Δ*Tffuc1*	*T. forsythia* knockout of the *Tffuc1* gene; Erm^r	This study
pET-22b (+)	Expression vector with a His_6-tag, Amp^r	Novagen
pJET1.2-*Tffuc1*_ko	pJET1.2 carrying the *Tffuc1_ermF-AM* knockout cassette	This study

Table 4. Oligonucleotide primers used for PCR amplification reactions[a]

Primers	Sequence (5' → 3')[a]
1	gcggCATATGAAAACAAGAACATTACTTCTTTGTG
2	gctaCTCGAGGTTTAGAGGCAATTCATTGGCAAATG
3	GACCAAGCTGCAGGCCATCATCGATGTGCTCAAC
A	*GAAGCTATCGGGGGTACCTCCCCCGGG*-AGAATAATTTTTGTTTATTACTAAAAAATAACG
5	*GCTTCGGGGATCCTCTAGCCCCCGGG*- CAGAAATATCTTTATGAAACATCCTATTGATGGGGTG
6	GCTCAGCCAGCCGATAGTTACTTTTTTCGTTATGTGTTCCC
7[b]	**CGTTATTTTTTAGTAATAAACAAAAATTATTCT-**CCCGGGGGAGGTACCCCCGATAGCTTC
8[b]	**CACCCCATCAATAGGATGTTTCATAAAGATATTTCTG-**CCCGGGGGCTAGAGGATCCCCGAAGC
9	CACGATGAACGTGTCGGTCATTAAC
10	GAAGCTATCGGGGGTACCTCCCCCGGG
11	GCTTCGGGGATCCTCTAGCCCCCGGG
12	GCACATATTTAGTAACCCGATAGCC

a. Artificial restriction sites are underlined. Lowercase letters indicate artificially introduced bases to improve restriction enzyme cutting. In italics are the overlap sequences complementary to *ermF-ermAM*. In bold are the overlap sequences complementary to the *BFO_2737* flanking regions.
b. Primer sequences were taken from Honma et al.[11]

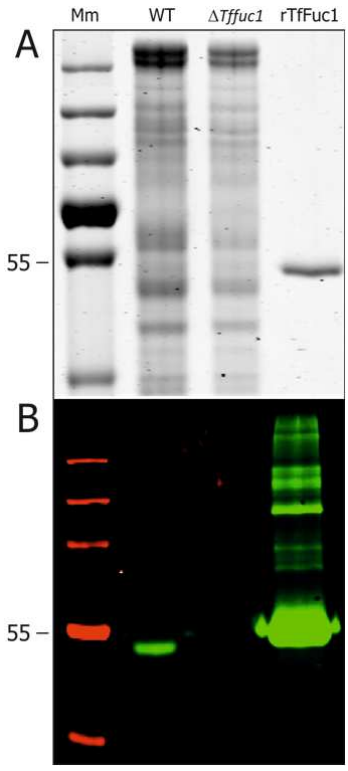

Figure 1. of Megson *et al.*

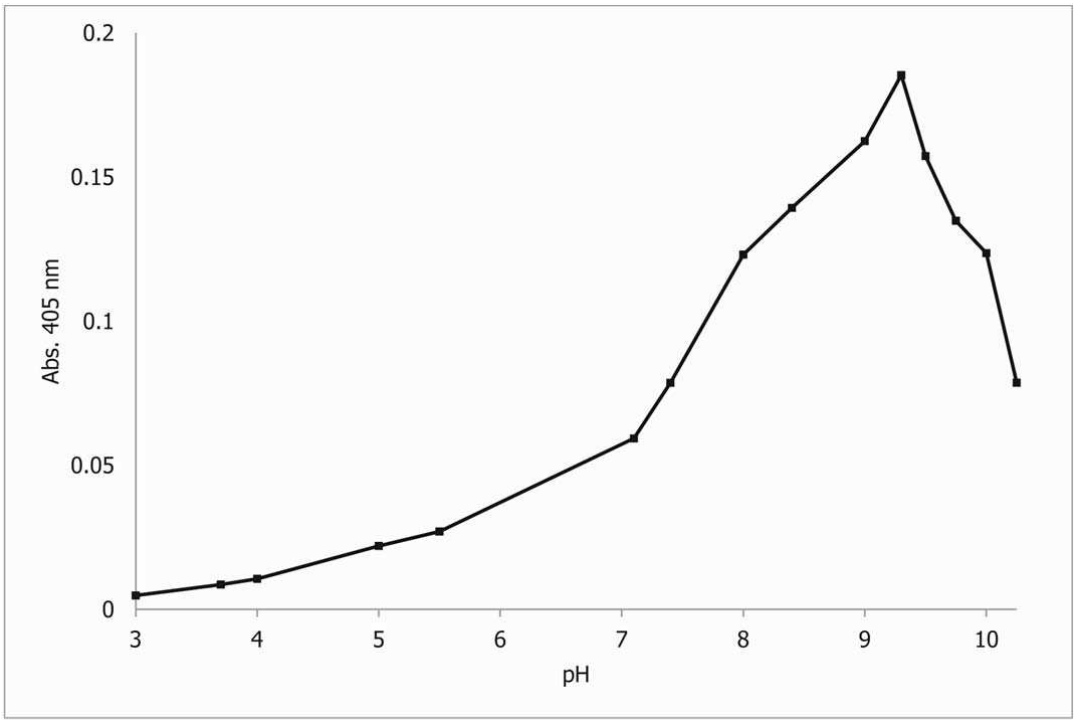

Figure 2. of Megson *et al.*

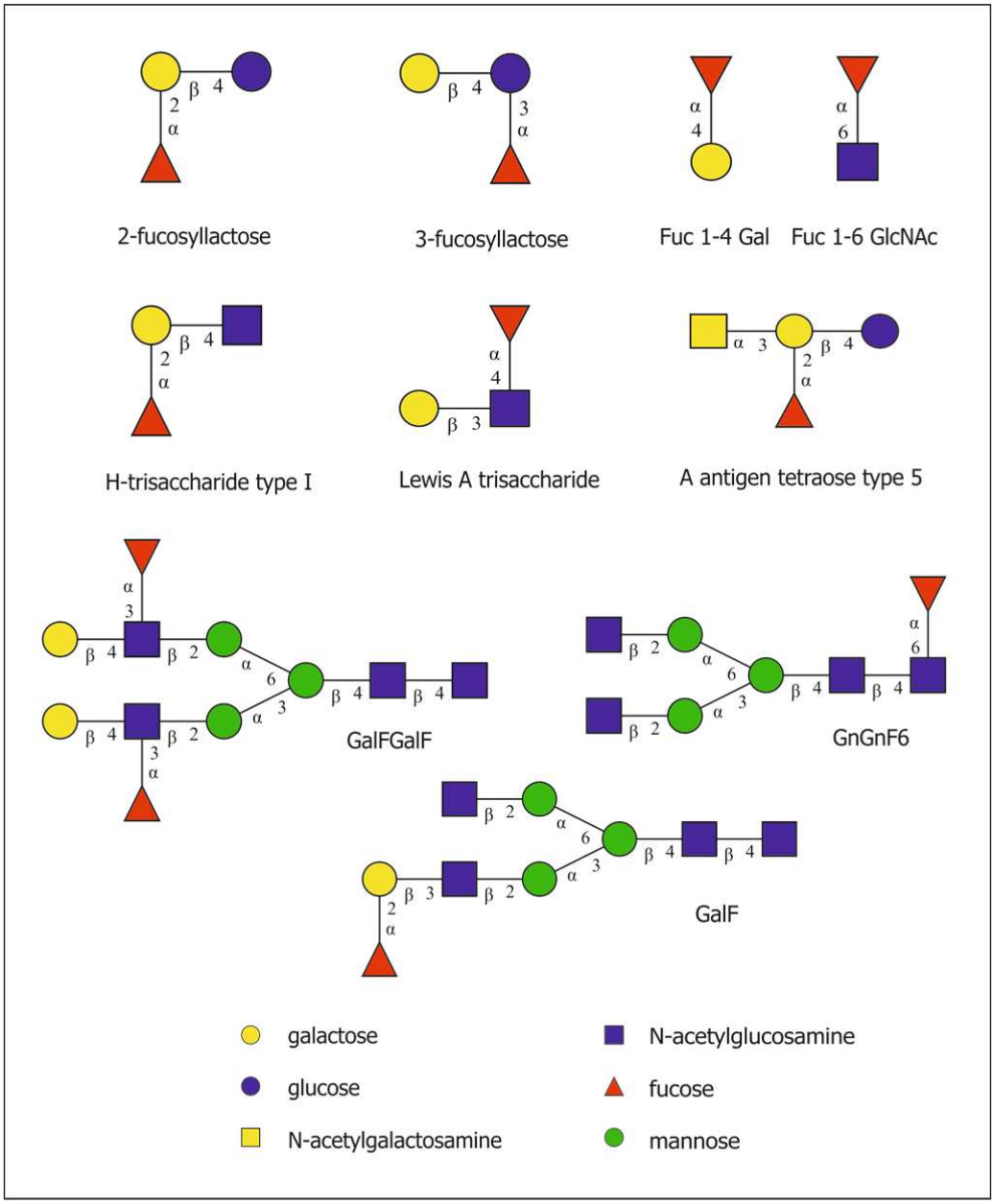

Figure 3. of Megson *et al.*

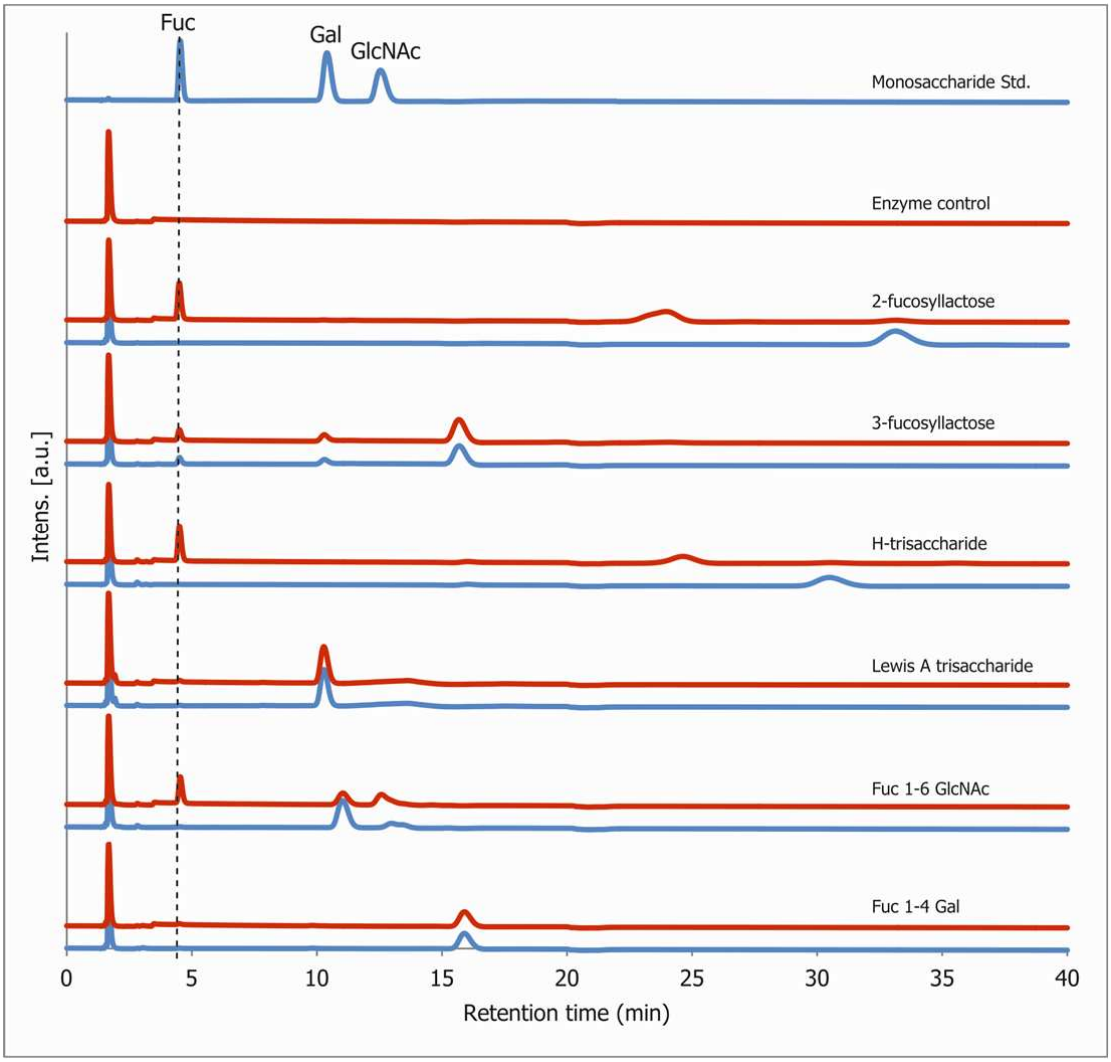

Figure 4. of Megson *et al.*

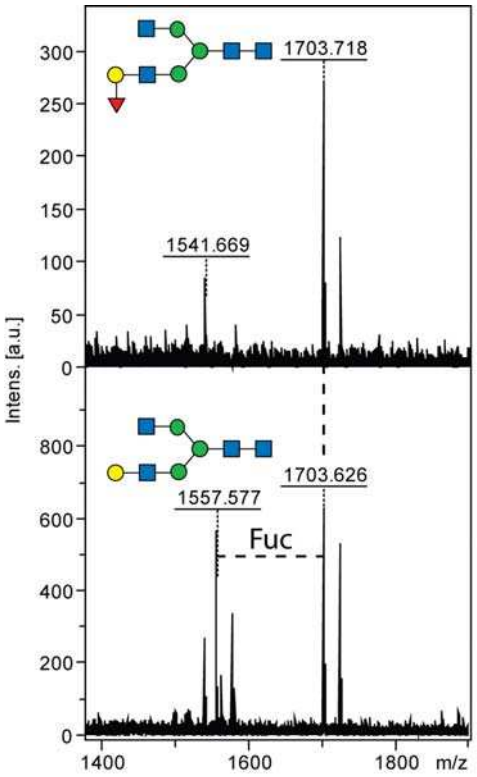

Figure 5. of Megson *et al.*

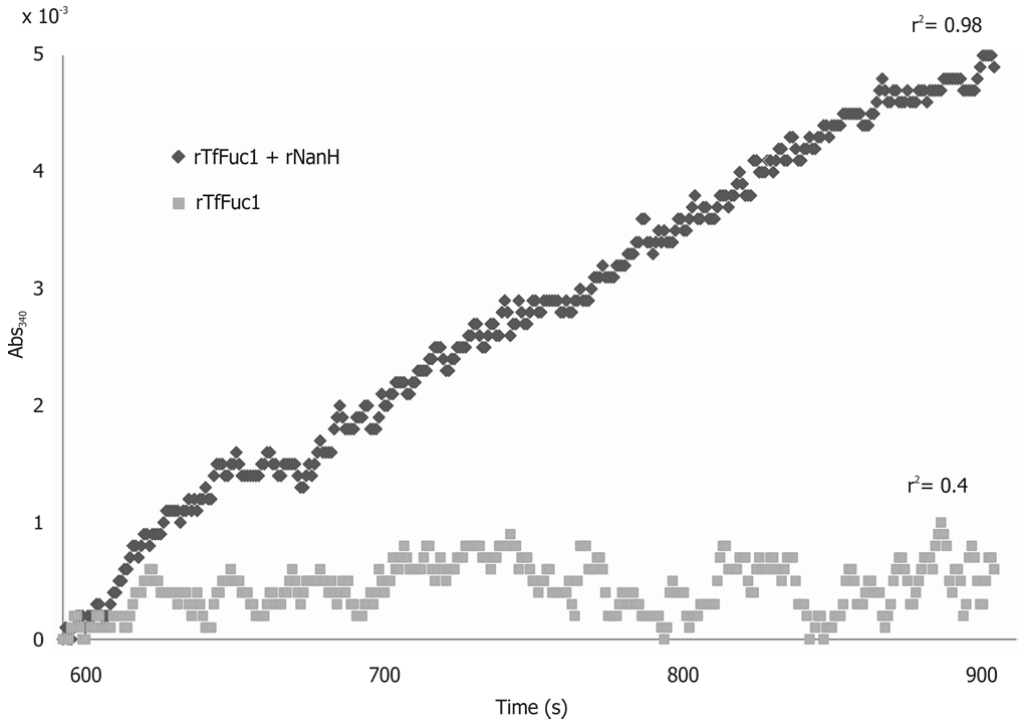

Figure 6. of Megson *et al.*

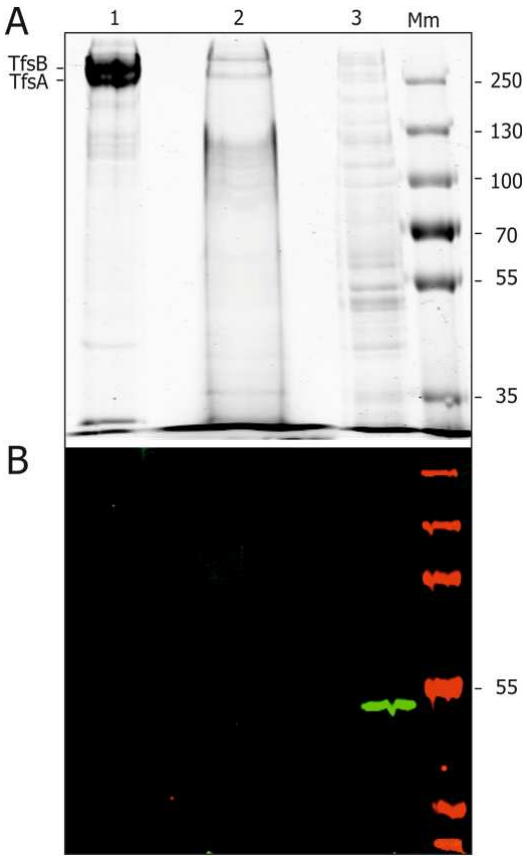

Figure 7. of Megson *et al.*

Submitted to Virulence

Research Paper

Characterization of an α-L-fucosidase from the periodontal pathogen *Tannerella forsythia*

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Key words: α(1,2) fucosidase; enzyme activity; enzyme specificity; oral pathogen; periodontitis; *Tannerella forsythia*

Abbreviations: Amp, ampicillin; BHI, brain heart infusion medium; CBB, Coomassie Brilliant Blue G 250; DFJ, deoxyfuconojirimycin; Erm, erythromycin; TfFuc1, *T. forsythia* ATCC 43037 fucosidase-1 encoded by the *bfo_2737* gene, equally *Tffuc1*; FDH, fucose dehydrogenase; HPAEC, high-pH anion-exchange chromatography with pulsed amperometric detection; LC-ESI-MS, liquid chromatography-electrospray ionisation-mass spectrometry; rTfFuc-1, recombinant TfFuc1 enzyme; NAM, *N*-acetylmuramic acid; PBS, phosphate-buffered saline; pNP-fucose; 4-nitrophenyl-α-L-fucopyranoside; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; *T. forsythia*, *Tannerella forsythia* ATCC 43037; WT, wild-type bacterium

Abstract

The periodontal pathogen *Tannerella forsythia* expresses several glycosidases which are linked to specific growth requirements and are involved in the invasion of host tissues. α-L-fucosyl residues are exposed on various host glycoconjugates and, thus, the α-L-fucosidases predicted in the *T. forsythia* ATCC 43037 genome could potentially serve roles in host-pathogen interactions. We describe the molecular cloning and characterization of the putative fucosidase TfFuc1 (encoded by the *bfo_2737* = *Tffuc1* gene), previously reported to be present in an outer membrane preparation. In terms of sequence, this 51-kDa protein is a member of the glycosyl hydrolase family GH29. Using an artificial substrate, p-nitrophenyl-α-fucose (K_M 670 μM), the enzyme was determined to have a pH optimum of 9.0 and to be competitively inhibited by fucose and deoxyfuconojirimycin. TfFuc1 was shown here to be a unique α(1,2)-fucosidase that also possesses α(1,6) specificity on small unbranched substrates. It is active on mucin after sialidase-catalysed removal of terminal sialic acid residues and also removes fucose from blood group H. Following knock-out of the *Tffuc1* gene and analysing biofilm formation and cell invasion/adhesion of the mutant in comparison to the wild-type, it is most likely that the enzyme does not act extracellularly. Biochemically interesting as the first fucosidase in *T. forsythia* to be characterized, the biological role of TfFuc1 may well be in the metabolism of short oligosaccharides in the periplasm, thereby indirectly contributing to the virulence of this organism. TfFuc1 is the first glycosyl hydrolase in the GH29 family reported to be a specific α(1,2)-fucosidase.

Introduction

Tannerella forsythia is a Gram-negative anaerobic oral pathogen, a member of the so-called "red complex" of bacteria that cause a set of inflammatory diseases named periodontitis, affecting millions of people worldwide.[1-3] The effects on the periodontium include loss of the alveolar bone around the teeth, swelling and bleeding of the gum and, in more severe cases, loss of teeth. Periodontitis has also been linked to systemic inflammation and to an increased risk of stroke, heart attacks and atherosclerosis, amongst others.[4]

Like other bacteria residing in human hosts, *T. forsythia* has adapted to better suit its niche with cell surface glycosylation thought to be key to this adaptation.[5] As previously described, *T. forsythia* cells are completely covered by a unique surface (S-) layer formed by co-assembly of two different proteins both of which are highly *O*-glycosylated with an equally unique glycan.[6-8] Mutant strains lacking either the S-layer or glycan assembly and maturation genes, display phenotypes involving altered human cell attachment to host cells, biofilm formation, and disease progression.[9-11] In addition, the structure of the glycan partially imitates that of host glycoproteins, having a terminal sialic acid-like residue (precisely, a modified pseudaminic acid residue) and a terminal fucose, with the latter shown to be present in substoichiometric amounts and linked to a methylated galactose in an unknown glycosidic linkage.[8] The glycobiology of this pathogen, including its repertoire of glycosidases, seems to be key to its physiology and, potentially, its pathogenicity.[5, 12]

Recent evidence suggests that for several periodontal pathogens, but particularly for the "red complex" organism *T. forsythia*, sialic acid-containing host molecules play an important role *in vivo*.[13] Two different sialidases have been found in *T. forsythia*, SiaHI and NanH. In the case of SiaHI, its function is unclear. It is not a canonical sialidase (*i.e.,* not in the GH35 family), a *siaH1* mutant has no discernible phenotype, and experiments point to it being a periplasmic protein without any role in extracellular interactions.[14] These same studies

also indicated that mutants lacking the main *T. forsythia* sialidase NanH had hindered attachment and invasion of human oral epithelial cells. The enzyme was also seen to play an important role in biofilm growth on surfaces coated with salivary glycoproteins.[15] The *nanH* gene is located in a large cluster that contains all the genes required for sialic acid catabolism, which indicates that the cleaved sialic acid can additionally be taken up and utilized.[12, 13] This gene cluster also contains a β-hexosaminidase that may cleave sub-terminal residues after sialidase action and may also play a role *in vivo*.[15] Additionally, in a separate study, transcriptome analysis of the oral microbiome also showed up-regulation of the sialidase *nanH* mRNA in dental plaque.[16]

α-L-fucosyl residues are, like sialic acid, frequently located at a terminal position on various host glycoconjugates including blood groups, milk oligosaccharides, gastric and submaxillary mucin, and serum glycoproteins.[17, 18] Therefore, fucosidases in *T. forsythia* could potentially play similar roles to sialidases.[19] It has generally been shown that terminal fucose residues play important roles in mammalian cell-cell communication and also in their interaction with pathogenic bacteria; for instance, *Campylobacter jejuni* and *Helicobacter pylori* are known to bind certain fucosylated blood groups (*e.g.,* O-antigen) in order to mediate infection.[19-21] In addition, the ability to utilize available fucose provides many bacteria with a nutritional advantage and contributes to survival in a highly competitive ecosystem, such as the human body.[22, 23]

Characterization of α-L-fucosidases in *T. forsythia* could aid in the elucidation of the structure-function relationship of fucosylated host and bacterial surfaces in the virulence of oral pathogens. The genome of *T. forsythia* encodes three putative α-L-fucosidases, BFO_2737 and BFO_1182, both classified in the CAZy (Carbohydrate Active enZymes; http://www.cazy.org/) glycosyl hydrolase family GH29, and BFO_3101, classified in family GH95. While all three enzymes possess a glycosyl hydrolase domain and are classified by CAZy according to their mechanism of action, BFO_1182 and BFO_3101 are not strictly

annotated as α-L-fucosidases but as a F5/8 type C domain protein and a putative lipoprotein, respectively.

Here, we describe the molecular cloning and characterization of BFO_2737, which we named TfFuc1. This protein has previously been reported to form part of an outer membrane preparation of *T. forsythia*[24] and, thus, was a good candidate to be involved in host-pathogen interactions. TfFuc1 is a 446-amino acid protein with a theoretical pI and molecular mass of 6.9 and 50.8 kDa, respectively. It is the first fucosidase in this organism to be characterized to date. The enzyme was shown here to be an α(1,2)-fucosidase and also possesses an α(1,6) specificity on small unbranched substrates. It is a predicted periplasmic protein, possibly playing a role in the breakdown of small oligosaccharides. It is, to the best of our knowledge, the first glycosyl hydrolase in its family (GH29) reported to be a specific α(1,2)-fucosidase.

Results

Enzymatic characterization of rTfFuc1. The *Tffuc1* gene was cloned into pET22-b(+) vector and expressed in *E. coli* as a C-terminally His_6-tagged protein, which enabled purification via nickel affinity chromatography (**Fig. 1**). The enzymatic activity was then tested using the standard colorimetric α-fucosidase substrate 4-nitrophenyl-α-L-fucopyranoside (pNP-fucose) at 22°C in a range of different pH values and in the presence of $MgCl_2$, KCl and NaCl, in order to establish its pH optimum and cation dependence, respectively. By stopping the reaction with the addition of an alkaline buffer at pH 11.4, it was ensured that all wells where at the same pH for consequent absorbance readings. The activity of the enzyme was seen to start to plateau at the neutral to alkaline pH range and was considered most active at pH 9.0, assayed in glycine buffer, and not at pH 9.25 where the activity suddenly peaks and then rapidly decreases thereafter. The activity remained largely unaffected by the presence of cations at the two concentrations tested (results not shown). The

K_M and V_{max} catalytic constants at 22°C, calculated from the activity of the enzyme at different pNP-fucose concentrations, were 670 μM and 20.4 μmol/min (U) per mg of protein, respectively (**Table 1**). The determined catalytic constants for rTffuc1 are in the range of those reported for other fucosidases/ glycosylhydrolases when tested on their corresponding pNP-substrates.[25-27]

Substrate linkage specificity of rTfFuc1. To determine the enzyme linkage specificity, rTfFuc1 was incubated with a set of different fucosylated substrates of defined structure representing a range of fucose linkages available on host glycoproteins and on oral surfaces (**Fig. 3**). The reaction products obtained after overnight incubation were analysed using high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC) where the release of fucose was confirmed by comparison with the retention time of the standard monosaccharide and of a substrate standard after overnight incubation at 37°C. The enzyme was seen to be active on both α(1,2) fucose containing substrates, 2-fucosyllactose and H-trisaccharide, and on the α(1,6) fucose disaccharide α-L-Fuc-(1,6)-β-D-GlcNAc, although this latter reaction did not reach completion, indicating weak specificity for this linkage. The α(1,3) and α(1,4) linkages were not cleaved on 3-fucosyllactose and the Lewis A trisaccharide, respectively. The enzyme was also inactive on the substrate α-L-Fuc-(1,4)-β-D-Gal, added as a second disaccharide control to prove that the α(1,6) activity was not due to differences in substrate length (**Fig. 4**).

In order to obtain accurate activity values on the cleaved substrates, the K-FUCOSE kit from Megazyme was used, coupled to the enzymatic reaction with rTfFuc1. First, FDH, which also has an alkaline pH optimum, and $NADP^+$ were added to the substrate solution reaction mixture in order to convert any free fucose already present in the sample to L-fucono-1,5-lactone by the reduction of $NADP^+$ to NADPH ($\varepsilon_{340} = 6.022\ mM^{-1}cm^{-1}$). rTfFuc1 was then added to the mixture and the reaction was monitored by following the increase in Abs_{340}. The

activity was calculated from where the formation of NADPH was linear over time. The enzyme was most active on 2-fucosyllactose and H-trisaccharide with specific activities of 0.8 U/mg and 0.6 U/mg, respectively. The activity on the α(1,6) disaccharide was significantly lower at 0.35 U/mg (**Table 2**).

The activity of the enzyme on the various substrates could be calculated approximately (as some loss of material occurred during sample preparation) from the HPLC experiments after 1-h incubation periods (results not shown) and was found to be markedly lower than that observed with the K-FUCOSE kit, indicating that free fucose, which is consumed in the latter, could be inhibiting the enzymatic activity significantly. In order to determine the extent of such an effect, measurement of K_M and V_{max} values were repeated with pNP-fucose in the presence of either 0.25 mM L-fucose or 0.1 μM deoxyfuconojirimycin (DFJ), which is a strong fucosidase inhibitor.[28] The enzyme was competitively inhibited by both fucose and DFJ as the V_{max} remained largely unaffected but the K_M value increased from 0.67 mM to 16.5 mM and 28.3 mM, respectively (**Table 1**).

Further, the ability of the enzyme to cleave fucose residues off more complex natural glycans and those on branched sugar residues was assayed (compare with **Fig. 3**). As expected, the enzyme was unable to cleave the α(1,3) fucose linkage on GalFGalF-pep, included in the assays as a trace amount of activity could be observed when using 3-fucosyllactose, as measured by the K-FUCOSE kit. The enzyme was also not able to cleave the core α-1,6 fucose linkage on $GnGnF^6$-pep nor the branched α(1,2)-linked fucose on the A antigen. The non-branched α(1,2) fucose linkage present on the Eastern oyster substrate, however, was cleaved off the substrate GalF, seen by the loss of a fucose residue in the MS spectra of the substrate. The major m/z 1703 glycan ($[M+H]^+$) was approximately 50% digested to a defucosylated species of m/z 1557 after overnight incubation with the enzyme (**Fig. 5**). The enzyme is, therefore, able to cleave off fucose residues which are α(1,2) linked on more complex glycans only when in a terminal unbranched position and is unable to cleave

core α(1,6) fucose. This data supports that the enzyme acts as an α(1,2) fucosidase.

rTfFuc1 activity on bovine submaxillary mucin. rTfFuc1 was incubated with mucin from bovine submaxillary glands and the release of fucose was measured with the K-FUCOSE kit. Incubation was performed also in combination with rNanH from *T. forsythia* and activities were calculated from the slope of ΔAbs_{340} where it was linear over time. During the assayed incubation period of 10 min, no activity could be detected when rTfFuc1 was incubated alone with mucin. The ΔAbs_{340} lead to an irregular data set with a very low r^2 value. However, fucose release was detectable when the incubation was performed in conjunction with the rNanH sialidase, presenting a slow but steady increase in the Abs_{340}. The activity was calculated over a period of 300 s where the data points fitted a linear regression with an r^2 of 0.98 (**Fig. 6**). The experiment was repeated several times and yielded an activity of 24 ±4 mU/mg. rTfFuc1 might cleave fucose off mucin over longer periods of time, but the data shows a significantly higher activity when sialic acid residues are first removed from mucin, indicating that the fucosidase TfFuc1 could work downstream from the sialidase in *T. forsythia* and presumably cooperate with other glycosidases in the degradation of complex glycans.

Cellular localization of TfFuc1. TfFuc1 was previously reported to be present in the outer membrane fraction of *T. forsythia*.[24] In an effort to investigate its presence on the surface of *T. forsythia* cells, TfFuc1-specific polyclonal antiserum was raised against the recombinant enzyme in mice and used for Western immunoblotting of cellular fractions separated by SDS-PAGE. Protein visualization by CBB staining showed good separation between the fractions, as the S-layer bands were very prominent in the outer membrane fraction but not in the inner-membrane and non-membrane associated fractions. Western immunoblotting showed that all the detectable TfFuc1 fucosidase was found in the non-membrane associated fraction

comprising both the cytoplasmic and periplasmic content (**Fig. 7**), arguing against surface localization of the TfFuc1 enzyme.

Discussion

Colonization of the periodontal pocket by the pathogenic late colonizer *T. forsythia* depends largely on pre-existing bacteria that have already tipped the oral balance away from health and towards disease.[29] Factors such as a pH shift from neutral to alkaline and slight raises in the temperature due to the host inflammatory response could be contributing factors favouring the process.[30] In a situation of oral disease, the number of different bacteria living in the gingival crevice decreases markedly due to putative pathogenic bacteria being more competitive in such an environment.[31] It is in these conditions that *T. forsythia* seems to thrive and becomes one of the key players in severe cases of periodontitis.

The NanH sialidase in *T. forsythia* has been well established to play important roles in adherence to sialylated glycoprotein-coated surfaces and epithelial cells in addition to triggering biofilm growth and being up-regulated in dental plaque.[14-16] As the other important terminal sugar on host glycoproteins is fucose, here, we performed an initial characterization of a putative α-L-fucosidase encoded in the *T. forsythia* genome, product of the gene *Tffuc1*, previously reported to be part of the outer membrane proteome.[24]

By producing the enzyme recombinantly in *E. coli* we were able to show that the enzyme is active across a broad pH range from 7.0-9.0, having an unusually high pH optimum of 9.0. It presents a unique α(1,2)-linkage specificity on terminal non-branched fucose residues, being also active on small non-branched α(1,6) fucosylated substrates. Whilst both these linkages are cleaved at a considerable rate in the case of small linear substrates, the α(1,6) specificity is not detected on core fucoses on more complex glycopeptides. The α(1,2) linkage specificity was apparent on both small linear substrates, such as 2-fucosyllactose and

H-trisaccharide, and on more complex glycans only when fucose occupied a terminal position, but not on a branched substrate where the fucose residue is linked to a fully substituted sugar. The enzyme seems to be, to the best of our knowledge, the first fucosidase in its GH family (GH29) to have a specific α(1,2) activity. The broad, high pH activity profile of this fucosidase ties in with its physiological niche which is known to have a pH that rises as periodontal disease progresses.[30] The possession of such enzymes with higher activities in alkaline surroundings could contribute to competiveness and virulence of *T. forsythia* in a diseased environment.

During the course of this study, it became clear that one of the issues possibly underlining our observations was the enzyme's cellular localization. Even though TfFuc1 was found previously to be present in the outer membrane proteome of the pathogen,[24] localization of the enzyme on the surface of *T. forsythia* cells by fluorescent immunolabelling was not successful (data not shown; see Experimental Procedures in the Supplementary Information) and cell fractionation also showed the detectable protein to be found in the non-membrane associated fraction (**Fig. 7**). This would be in agreement with bioinformatics interrogation indicating that, while it has a Sec-dependent secretion signal (as predicted by SignalP 4.1 Server), it is not predicted to reside in the outer membrane or be secreted, nor does it contain a T9SS C-terminal secretion signal.[32] The difference in location of TfFuc1 between the present study and the outer membrane proteomics experiment[24] can be reconciled when considering that in the latter study, cross-contamination of individual proteins between cellular fractions was not investigated.

Incubation of the recombinant fucosidase with bovine submaxillary mucin showed no detectable release of fucose over an incubation period of 10 min. Activity on this complex substrate could only be detected when the incubation was performed in combination with the recombinant NanH sialidase from *T. forsythia*. It is, therefore, conceivable that TfFuc1 could play an accompanying role to the sialidase in the interaction between *T. forsythia* and host

glycoproteins, but given its periplasmic location, this could merely reflect the need for removal of terminal sialic acid residues for the enzyme to work, either indicating that it most likely acts on internalised fucosyl substrates after sialic acid has already been removed by the action of sialidases or, less likely, that it acts in concert with sialidases externally.

This notion that the *T. forsythia* fucosidase TfFuc1 plays an internal role was corroborated when we tested the effect of the Δ*Tffuc1* mutation on the ability of *T. forsythia* to interact with and invade human oral epithelial cells using an antibiotic protection assay on the oral epithelial cell line H357.[33] We found no significant differences in the ability of the Δ*Tffuc1* to invade these human cells as compared to the WT strain (**Fig. S1)**, indicating that TfFuc1 has no effect on epithelial cell-invasion under the conditions tested. In addition, the mutant did not show hindered biofilm formation when cultured on bovine submaxillary mucin, contrary to the *T. forsythia NanH* sialidase mutant,[15] but showed a slightly increased biofilm formation (**Fig. S2**).

Our data supports the idea of a periplasmic fucosidase involved in the final breakdown of small substrates that have been internalized, possibly owing to the action of exoglycosidases and endoglycosidases which break-down larger glycans on the outside of the cell.[34, 35] TfFuc1 would possibly then be able to exert its full potential freeing both α(1,2) and α(1,6) fucoses on small linear substrates. These findings are also in agreement with the hypothesis that the fucosidase acts downstream of the sialidases, which have been shown to act on whole glycoproteins on bacterial and host surfaces.[14, 15, 36] The sialic acid would, therefore, already be missing as smaller parts of the glycan are transported inside the cell.

T. forsythia has no straightforwardly identifiable fucose catabolism locus in its genome, nor does it have the bifunctional L-fucokinase/GDP-fucose pyrophosphorylase required normally for *Bacteroidetes* to recycle the fucose into its glycans.[23] In an effort to see the effect on the latter scenario, the fucose containing S-layer glycan from both the WT and the Δ*Tffuc1* strains were compared by LC-ESI-MS with no obvious change under the growth

conditions used (Z.A. Megson, L. Neumann, F. Altmann, C. Schäffer, unpublished data). However, the microheterogeneity of the S-layer glycan regarding the terminal fucose residue complicates interpretation of MS data. Therefore, it remains unclear whether the released fucose in the periplasm can be used as a nutrient source or is recycled by the bacteria into its glycosylation pathway, and, thus, is subject of further studies.

Overall, our data suggest that TfFuc1 is a unique α-L-(1,2)-fucosidase which could potentially contribute to fucose utilization in *T. forsythia*. In order to better elucidate this role and rule out any redundancy in the system, two further annotated fucosidases in *T. forsythia*, BFO_1182 and BFO_3101, together with the annotated fucose permease, BFO_0307, are now being investigated to elucidate the role of fucose in the physiology of *T. forsythia*.

Experimental Procedures

Bacterial strains, medium and culture conditions. *T. forsythia* wild-type (WT) strain ATCC 43037 (American Type Culture Collection) and the knock-out mutant Δ*Tffuc1* were grown anaerobically at 37°C for 4-7 d in brain heart infusion (BHI) broth or 0.8% (w/v) BHI agar, supplemented with *N*-acetylmuramic acid (NAM), horse serum and gentamycin as described previously.[32] *Escherichia coli* DH5α and BL21 (DE3) (Invitrogen) were cultivated in selective Luria Bertani (LB) medium (agar and broth) supplemented with 100 µg/ml ampicillin (Amp). All strains and plasmids used in the course of this study are summarised in **Table 3**.

Molecular methods. All enzymes were purchased from Fermentas. Genomic DNA of *T. forsythia* WT strain ATCC 43037 was isolated from 2 ml of bacterial suspension as described previously and used as the DNA template in all PCRs, unless otherwise specified.[37] The

GeneJET™ Gel Extraction Kit (Fermentas) was used to purify DNA fragments from agarose gels and to purify digested plasmids and oligonucleotides. Plasmid DNA from transformed cells was isolated with the GeneJET™ Plasmid Miniprep kit (Fermentas). Agarose gel electrophoresis was performed as described elsewhere.[38] Primers for PCR and DNA sequencing were purchased from Invitrogen (**Table 4**). PCR was performed using the Phusion®High-Fidelity DNA Polymerase (Fermentas) and a My CyclerTM (Bio-Rad) thermal cycler. Transformation of chemically competent *E. coli* DH5α and BL21 (DE) cells was performed according to the manufacturer's protocol (Invitrogen). *E. coli* transformants were screened by PCR using RedTaq ReadyMix PCR mix (Sigma-Aldrich) and recombinant clones were analyzed by restriction mapping. Expression vector and knock-out cassette were sequenced (Microsynth) prior to transformation.

Recombinant production of His_6-tagged TfFuc1. The *Tffuc1* gene was amplified from the chromosome of *T. forsythia* ATCC 43037 with a fused C-terminal His_6-tag by PCR using primer pair 1/2 (**Table 4**). The His_6-tagged amplification product was digested using restriction enzymes NdeI/XhoI and cloned into NdeI/XhoI-linearized pET22-b (Novagen). The corresponding plasmid was transformed into *E. coli* BL21 (DE3) cells for protein expression. Freshly transformed cells were grown in two 400-ml Erlenmeyer flasks to an OD_{600} of 0.4-0.5 in the presence of 100 µg/ml of Amp at which point protein expression was induced with a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and cultures were shaken (200 rpm) overnight at 18°C. Cells were harvested by centrifugation (6,500 *g*, 20 min, 4°C).

Collected bacterial cells were lysed by sonication in buffer A (50 mM phosphate buffer pH 8, 0.3 M NaCl) containing 5 mM imidazole and cleared lysates after ultracentrifugation at 150,000 *g* for 30 min at 4°C were incubated with 1 ml of Ni-NTA beads (Qiagen) for 1 h at 4°C, shaking slightly. The beads were placed in a chromatography column

and the His_6-tagged protein was purified using an imidazole gradient in buffer A; 25 mM imidazole (10 ml), 50 mM imidazole (10 ml), followed by five elution steps with 500 µl of 250 mM imidazole in buffer A. Eluted fractions containing the purified recombinant protein, rTfFuc1, as determined by SDS-PAGE analysis, were pooled and dialysed overnight at 4°C against 3 l of 10 mM phosphate buffer, pH 8.0. The volume was then reduced 5-fold using a concentration centrifuge yielding a protein concentration of 0.35 mg/ml (as determined by Nanodrop) in 50 mM phosphate buffer.

Construction of a *T. forsythia* Δ*Tffuc1* knock-out strain. Disruption of the *Tffuc1* gene in *T. forsythia* was performed by gene knockout, as described previously.[32] The *Tffuc1* gene is not part of an operon, thus, downstream effects due to the chosen mutation strategy are not expected to occur. Briefly, the flanking genomic regions (1,000 bp) up-stream and down-stream of *Tffuc1* were amplified using primer pairs 3/4 and 5/6, respectively (**Table 4**). The two resulting fragments were joined with the erythromycin resistance gene *ermF-ermAM* (amplified using primer pair 7/8) by overlap extension PCR and sub-cloned into the blunt-end cloning vector pJET1.2 (Thermo Scientific), resulting in pJET1.2/*Tffuc1*_ko. Approximately 5 µg of the knockout cassette was transferred by electroporation into 100 µl of competent *T. forsythia* cells. Cells were regenerated in BHI medium for 24 h before plating on BHI agar plates containing erythromycin (10 µg/ml) as a selection marker. Single colonies were picked and used for inoculation of liquid BHI medium. Genomic DNA of the new Δ*Tffuc1* mutants were isolated as mentioned above and the absence of the *Tffuc1* gene and the correct integration of the erythromycin resistance gene (upstream and downstream) was evaluated by PCR using primer pairs 1/2, 9/10, and 11/12, respectively (**Table 4**). Absence of the enzyme in the Δ*Tffuc1* strain was also confirmed by Western immunoblotting of the total cell extract separated by SDS-PAGE using TfFuc1-specific polyclonal antiserum (**Fig. 1**).

General and analytical methods. SDS-PAGE was carried out according to a standard protocol using a Protean II electrophoresis apparatus (Bio-Rad).[39] Protein bands were visualized with Coomassie Brilliant Blue G 250 (CBB) staining reagent. For Western immunoblotting of proteins onto a nitrocellulose membrane (Peqlab), a Mini Trans-Blot Cell (Bio-Rad) was used. Detection of the His_6-tag fused to rTfFuc1 and detection of TfFuc1 was done with the Li-Cor Odyssey Infrared Imaging System using an anti-His_6 mouse antibody (Roche) or TfFuc1-specific polyclonal antiserum raised in mice (EF-BIO), respectively, both in combination with goat anti-mouse IgGIR Dye 800CW conjugate (Li-Cor).

Enzymatic characterization of rTfFuc1. Enzymatic characterization of rTfFuc1 was performed essentially as described elsewhere.[40] 0.17 µM of purified, recombinant enzyme was incubated with 5 mM of the colorimetric substrate 4-nitrophenyl-α-L-fucopyranoside (pNP-fucose) (Sigma) at a range of different pH values (3-10.25) and cation concentrations in 96-well plates at 22°C in a total volume of 40 µl. The enzymatic reaction was stopped after 3 min by addition of 260 µl of phosphate buffer, pH 11.4. Citrate/phosphate buffer (0.1 M) was used to assay the pH range from 3-8,[41] 50 mM glycine buffer was used for a pH range from 8-10. The effect of $MgCl_2$ (5 mM, 10 mM), KCl (5 mM, 10 mM) and NaCl (50 mM, 150 mM) on the enzyme's activity was assayed in the same way in 50 mM glycine buffer, pH 9.

A 4-nitrophenol standard curve was made by measuring the absorbance at 405 nm (Abs_{405}) of 0, 4, 8, 12, 16, 20 and 24 nmol of 4-nitrophenol per well in 300 µl of phosphate buffer, pH 11.4. The K_M and V_{max} catalytic constants were calculated at pH 9.0 in 50 mM glycine buffer at 22°C in the presence of 0.01 to 50 mM pNP-fucose. The inhibitory effect of fucose and deoxyfuconojirimycin (DFJ) on the K_M and V_{max} of the enzyme were assayed in the same way in the presence of 0.25 mM fucose and 0.1 µM DFJ, respectively.[28] Readings were performed using an Infinite 200 plate reader (TECAN) and catalytic constants

were calculated with the Sigma Plot 12, Systat Software.

Substrate specificity of rTfFuc1. For the determination of enzyme linkage specificity, a set of commercially available fucosylated substrates (2-fucosyllactose and 3-fucosyllactose from Dextra laboratories; H-Trisaccharide, Lewis A trisaccharide, Fuc-α-(1,4)-Gal and Fuc-α-(1,6)-GlcNAc, all from Carbosynth) (**Fig. 3**) were incubated with the enzyme and reaction mixtures were analysed by HPAEC using an ICS3000 chromatographic system (Dionex, Thermo Fisher) on a CarboPac PA-1 column. Incubations were made overnight at 37°C in a total volume of 100 µl by mixing 0.34 µM of enzyme with 0.5 mM of substrate in 50 mM glycine buffer, pH 9.0. In order to minimize the effect of the buffer, the reaction volume was then diluted with 400 µl of Milli-Q water and the enzyme was removed using an Amicon 3 kDa cut-off spin column (Millipore). Twenty-five microliters of this flow through was then applied to the CarboPacPA-1 column using full-loop injection.

In order to obtain reliable activity values, the K-FUCOSE kit (Megazyme) was adapted to suit requirements. In a total volume of 250 µl of 50 mM glycine buffer at pH 9.0, the substrates 2-fucosyllactose, H-trisaccharide and Fuc-α(1,6)-GlcNAc, were incubated separately at a concentration of 0.5 mM with 1.83 µl of fucose dehydrogenase (FDH) and 9.15 µl of $NADP^+$ (both as supplied) in a cuvette at 37°C for 10 min. When the reaction had reached a constant absorbance at 340 nm (Abs_{340}), rTfFuc1 was added to the mixture at a concentration of 0.34 µM and the formation of NADPH was followed by continuous measurement of the increase in Abs_{340}. The activity of the enzyme on each substrate was calculated according to the supplier's specifications from ΔAbs_{340}/min where the formation of NADPH was linear over time. The experiment was repeated with different enzyme dilutions (1:10; 1:100) to prove the reliability of the method.

The ability of the enzyme to cleave fucose residues off more complex natural glycans and those on branched sugar residues was assayed on the substrates A antigen tetraose type 5

(Carbosynth), GnGnF^6-peptide, GalFGalF, and an *N*-glycan derived from *Crassostrea virginica* (Eastern oyster) haemocyte treated with chicken liver α-*N*-acetylgalactosaminidase and bovine β-galactosidase (both from Sigma) to reveal the underlying H epitope presenting a terminal α(1,2) fucose on an unsubstituted β(1,3)-galactose (*i.e.,* histo blood group antigen H), referred to here as GalF^{42} (**Fig. 3**). Activity on the A antigen substrate was assayed using the K-FUCOSE kit as described above. For all other substrates, incubations were performed overnight at 37°C and analysed by MALDI-TOF MS using an Autoflex Speed instrument (Bruker) in positive ion mode with 6-aza-2-thiothymine (ATT) as matrix. 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 then manually interpreted.

rTfFuc1 activity on mucin from bovine submaxillary glands. rTfFuc1 was incubated with bovine submaxillary mucin (Sigma) in combination with the His-tagged recombinant NanH sialidase (rNanH) from *T. forsythia*.[15] Incubations were performed at 37°C in 50 mM glycine buffer, pH 9.0, at a final concentration of 0.1 µM of either enzyme, 0.2 mg/ml of mucin and $NADP^+$ and FDH as described above, in a total volume of 250 µl. rTfFuc1 (and rNanH) was added to the mixture after an initial 5-min incubation period. The release of fucose (ΔAbs_{340}) was followed over 10 min and calculated according to the supplier's specifications from ΔAbs_{340}/min where the formation of NADPH was linear over time.

Presence of TfFuc1 in *T. forsythia* membrane, outer membrane and non-membrane preparations. Cells were harvested by centrifugation from a 4-day-old 100-ml *T. forsythia* culture. Separation of cellular fractions was performed as described previously.[24] Briefly, cells were washed once in Tris (2-amino-2-hydroxymethyl-propane-1,3-diol)-buffer, pH 7.5, sonicated, and cell debris were removed by centrifugation. The collected supernatant was

ultracentrifuged (100,000 *g*, 4°C, 40 min) to separate the whole membrane fraction (pellet) from the membrane non-associated fraction (cytoplasm and periplasm, supernatant). The pellet was resuspended in 2% (w/v) N-lauroylsarcosine (Sigma) in Tris buffer and mixed. After incubation (2 h, 25°C), the outer membrane fraction (OM) was collected by centrifugation (100,000 *g*, 4°C, 40 min) and the pellet was resuspended in Tris buffer. The protein content was determined in each fraction by the Bradford method (Bio-Rad).[43] A total of 20 µg of protein from the OM and non-membrane associated fractions and 400 µg of the membrane fraction was loaded onto an SDS-PAGE gel and ran as described above. The presence of TfFuc1 in each fraction was determined by Western immunoblotting.

Microtiter assays of Hoechst-stained biofilms. *T. forsythia* WT and Δ*Tffuc1* strains were compared in respect to the biofilm formation[44] on mucin-coated polystyrene microtiter plates in dependency of the strength of the BHI liquid medium. Biofilm was stained with Hoechst 33258 Fluorescent Stain (Thermo Scientific). Details are described in the Supplementary Information.

Attachment and invasion assays. For both attachment and invasion assays, *T. forsythia* WT and Δ*Tffuc1* strains were incubated with the oral epithelial cell line H357 (CCL17; American Type Culture Collection) at a multiplicity of infection of 1:100, as described previously.[45] Details are described in the Supplementary Information

Disclosure and Potential Conflict of Interest

No potential conflicts of interests were disclosed.

Acknowledgements

The authors thank Dr. Bernard Henrissat (CAZy) for discussing classification of the TfFuc1 enzyme and Sonja Zayni for excellent technical assistance with HPAEC sugar analysis. This work was supported by the Austrian Science Fund FWF project P24317-B22 (to C. S.) and the Doctoral Programme Biomolecular Technology of Proteins (FWF project W1224).

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Figure legends

Figure 1. SDS PAGE (**A.**) and Western immunoblot (**B.**) of total cell extracts from *T. forsythia* WT (lane 2) and Δ*Tffuc1* strains (lane 3) and of the His_6-tagged rTfFuc1 as purified from *E. coli* (lane 4), used for activity studies and to raise a polyclonal anti-TfFuc1 antiserum. Western immunoblotting using the anti-TfFuc1 antiserum recognized the protein (~51 kDa) specifically in the WT strain (lane 2) and indicated absence of the protein in the Δ*Tffuc1* strain (lane 3), proving that the enzyme was effectively knocked-out. In the preparation of rTfFuc 1 (B., lane 4), the polyclonal antiserum recognizes also minor contaminating *E. coli* proteins not visible on the SDS-PAGE gel (A., lane 4). Mm; PageRuler Plus prestained protein ladder (Thermo Scientific).

Figure 2. pH profile of rTfFuc1 using 4-nitrophenyl-α-L-fucopyranoside (pNP-fucose) as a substrate. Activity was measured as the increase in Abs_{405} due to the released 4-nitrophenol product. Citrate/phosphate buffer (0.1 M) was used to assay the pH range from 3-8, 50 mM glycine buffer was used for the pH range from 8-10.25.

Figure 3. Fucosylated substrates used in this study. The structures are depicted according to the symbolic nomenclature of the Consortium for Functional Glycomics (http://www.functionalglycomics.org/static/consortium/Nomenclature.shtml).

Figure 4. rTfFuc1 activity on standard fucosylated substrates after overnight incubation as determined by HPAEC. Blue lines represent samples which were incubated in absence of rTfFuc1 (substrate standard) and red lines represent samples incubated in the presence of rTfFuc1. Cleavage of the substrates was determined by the appearance of a fucose peak, as determined by the retention time of the standard monosaccharide.

Figure 5. Cleavage of natural α(1,2) fucosylated glycans by rTfFuc1**.** Cleavage of fucose from a large *N*-glycan substrate was monitored by MALDI-TOF MS spectra after overnight incubation; the conversion of the *m/z* 1703 glycan (GalF) to one of *m/z* 1557 (*Δm/z* 146) is indicative of the loss of fucose. The structures of the substrate and product are depicted according to the symbolic nomenclature of the Consortium for Functional Glycomics.

Figure 6. rTfFuc1 was incubated with mucin from bovine submaxillary glands and the release of fucose was measured with the K-FUCOSE kit. When incubations were performed in conjunction with the rNanH sialidase, a slow steady increase in the Abs_{340} was observed. The activity was calculated over a period of 300 s where the data points fitted a linear regression with an r^2 of 0.98. No activity could be detected, when rTfFuc1 was incubated alone with the mucin. The ΔAbs_{340} lead to an irregular data set with a very low r^2 value of 0.4.

Figure 7. Presence of TfFuc1 in cell fractions of *T. forsythia* WT. **A.** SDS-PAGE analysis of the outer membrane fraction (OM) (1), membrane fraction (2) and non-membrane associated fraction (3) showed good separation between the fractions, as the S-layer bands were very prominent in the OM but not in the membrane and non-membrane associated fractions. Protein loaded was 20 µg of the OM and non-membrane associated fractions and 400 µg of the membrane fraction. Protein visualization was by CBB. **B.** Western immunoblot using anti-TfFuc1 antiserum showed the TfFuc1 fucosidase in the non-membrane associated fraction comprising both the cytoplasmic and periplasmic content. Mm; PageRuler Plus prestained protein ladder (Thermo Scientific).

Table 1. rfFuc1 activity on 4-nitrophenyl-α-L-fucopyranoside (pNP-fucose). Catalytic constants K_M and V_{max} and the inhibitory effect of DFJ and L-fucose were measured using the colorimetric substrate pNP-fucose within a concentration range from 0.01 to 50 mM at 22°C in glycine buffer at pH 9.0

Substrate*	Inhibitor	K_M (mM)	V_{max} (U/mg)**
pNp-fucose	None	0.67 (±0.2)	20.4 (±0.8)
pNp-fucose	0.1 µM DFJ***	28.3 (±3.7)	28.1 (±2.4)
pNp-fucose	0.25 mM Fucose	16.5 (±4.7)	28.6 (±4.4)

*4-nitrophenyl-α-L-fucopyranoside

**µmol/min/mg of enzyme

***deoxyfuconojirimycin

Table 2. rTfFuc1 activity on standard fucosylated substrates. Cleavage was determined by HPAEC after overnight incubations with rTfFuc1 (see **Fig. 4**, with the exception of A antigen tetraose) and specific activities were calculated using the K-FUCOSE kit

Substrate	Structure	Fucose linkage	Enzyme cleaves	Activity (U/mg)*
2-Fucosyllactose	α-L-Fuc-1,2-β-D-Gal-1,4-D-Glc	α(1,2)	Yes	0.8
3-Fucosyllactose	β-D-Gal-1,4(α-L-Fuc-1,3)-D-Glc	α(1,3)	No	-
H-trisaccharide	α-L-Fuc-1,2-β-D-Gal-1,3-β-D-GlcNAc	α(1,2)	Yes	0.6
Lewis A trisaccharide	β-D-Gal-1,3(α-L-Fuc-1,4)-β-D-GlcNAc	α(1,4)	No	-
Fuc(1,6)GlcNAc	α-L-Fuc-1,6-β-D-GlcNAc	α(1,6)	Yes	0.35
Fuc(1,4)Gal	α-L-Fuc-1,4-β-D-Gal	α(1,4)	No	-
A antigen tetraose	β-GalNAc-1,3(α-L-Fuc-1,2)-β-D-Gal-1,4-D-Glc	α(1,2)	No	-

*μmol/min/mg of enzyme

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant characteristic(s)	Source
Escherichia coli DH5α	F$^-$ Φ80d*lacZ* M15 (*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (rK$^-$mK$^-$) *phoA supE44 thi-1 gyrA96 relA1*$^-$	Invitrogen
Escherichia coli BL21 (DE)	*F*$^-$*, ompT, hsdS* (*rB*$^-$*mB*$^-$), *gal, dcm* (DE3)	Invitrogen
Tannerella forsythia ATCC 43037	Wild-type isolate	American Type Culture Collection, USA
T. forsythia Δ*Tffuc1*	*T. forsythia* knockout of the *Tffuc1* gene; Erm^r	This study
pET-22b (+)	Expression vector with a His_6-tag, Amp^r	Novagen
pJET1.2-*Tffuc1*_ko	pJET1.2 carrying the *Tffuc1_ermF-AM* knockout cassette	This study

Table 4. Oligonucleotide primers used for PCR amplification reactions[a]

Primers	Sequence (5' → 3')[a]
1	gcggCATATGAAAACAAGAACATTACTTCTTTGTG
2	gctaCTCGAGGTTTAGAGGCAATTCATTGGCAAATG
3	GACCAAGCTGCAGGCCATCATCGATGTGCTCAAC
A	*GAAGCTATCGGGGGTACCTCCCCCGGG*-AGAATAATTTTTGTTTATTACTAAAAAATAACG
5	*GCTTCGGGGATCCTCTAGCCCCCGGG*- CAGAAATATCTTTATGAAACATCCTATTGATGGGGTG
6	GCTCAGCCAGCCGATAGTTACTTTTTTCGTTATGTGTTCCC
7[b]	**CGTTATTTTTTAGTAATAAACAAAAATTATTCT-**CCCGGGGGAGGTACCCCCGATAGCTTC
8[b]	**CACCCCATCAATAGGATGTTTCATAAAGATATTTCTG-**CCCGGGGGCTAGAGGATCCCCGAAGC
9	CACGATGAACGTGTCGGTCATTAAC
10	GAAGCTATCGGGGGTACCTCCCCCGGG
11	GCTTCGGGGATCCTCTAGCCCCCGGG
12	GCACATATTTAGTAACCCGATAGCC

a. Artificial restriction sites are underlined. Lowercase letters indicate artificially introduced bases to improve restriction enzyme cutting.
In italics are the overlap sequences complementary to *ermF-ermAM*. In bold are the overlap sequences complementary to the *BFO_2737* flanking regions.
b. Primer sequences were taken from Honma et al.[11]

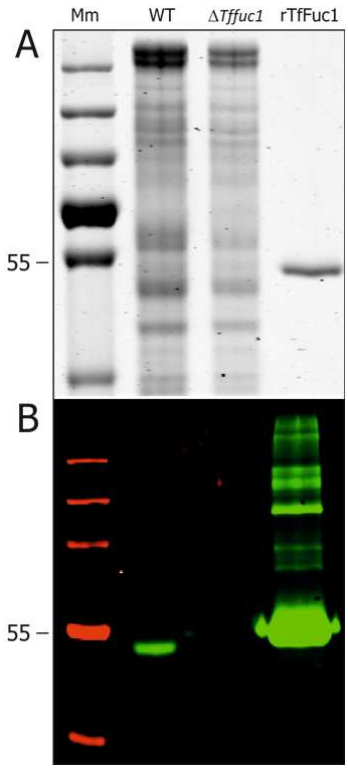


Figure 1. of Megson *et al.*

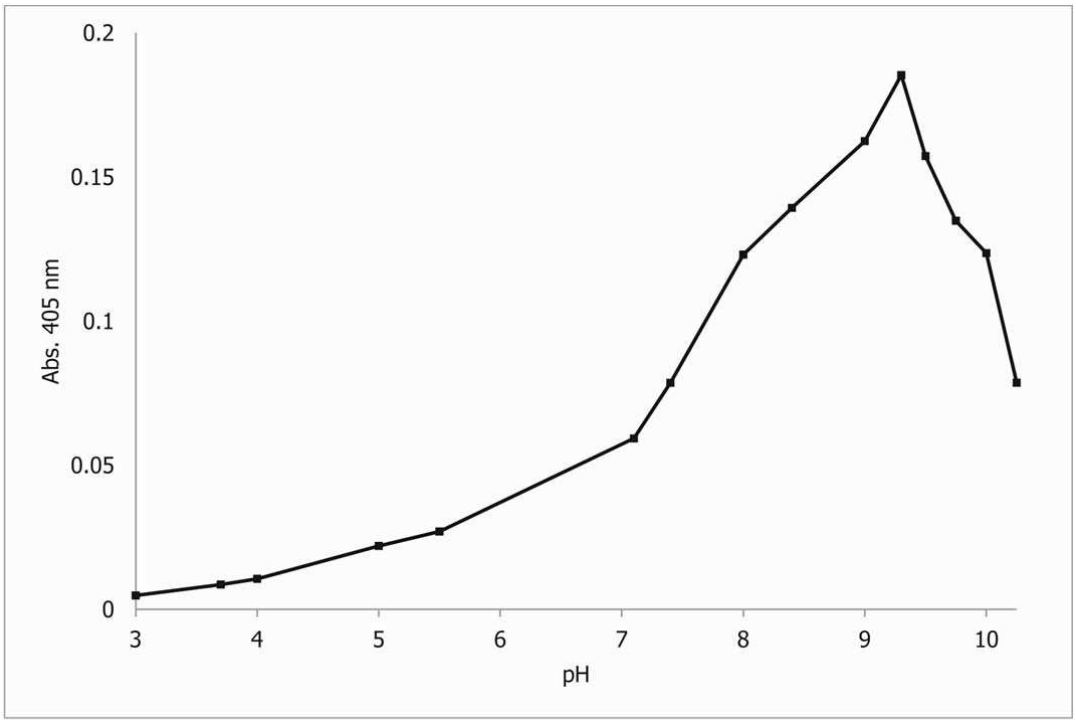


Figure 2. of Megson *et al.*

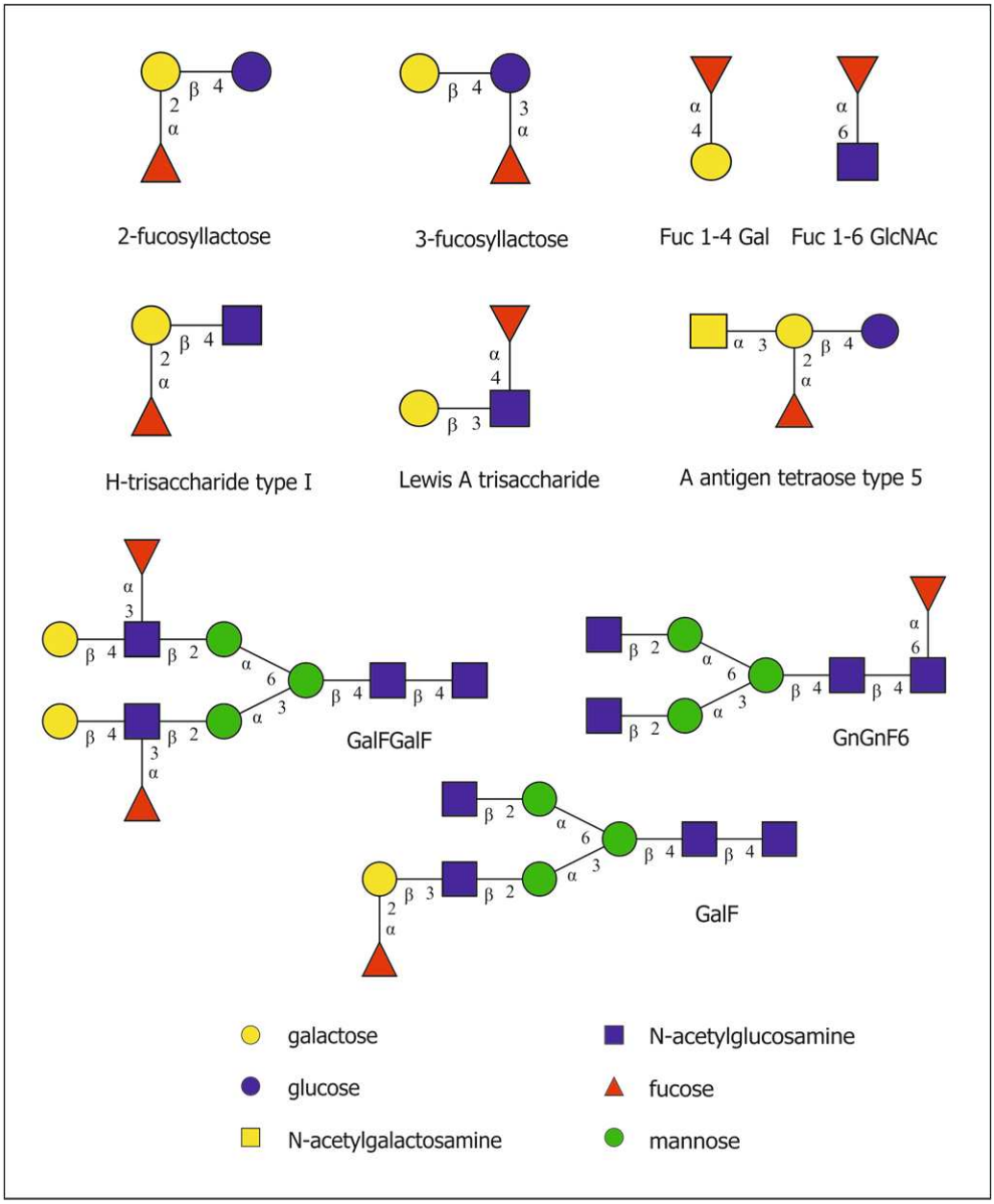


Figure 3. of Megson *et al.*

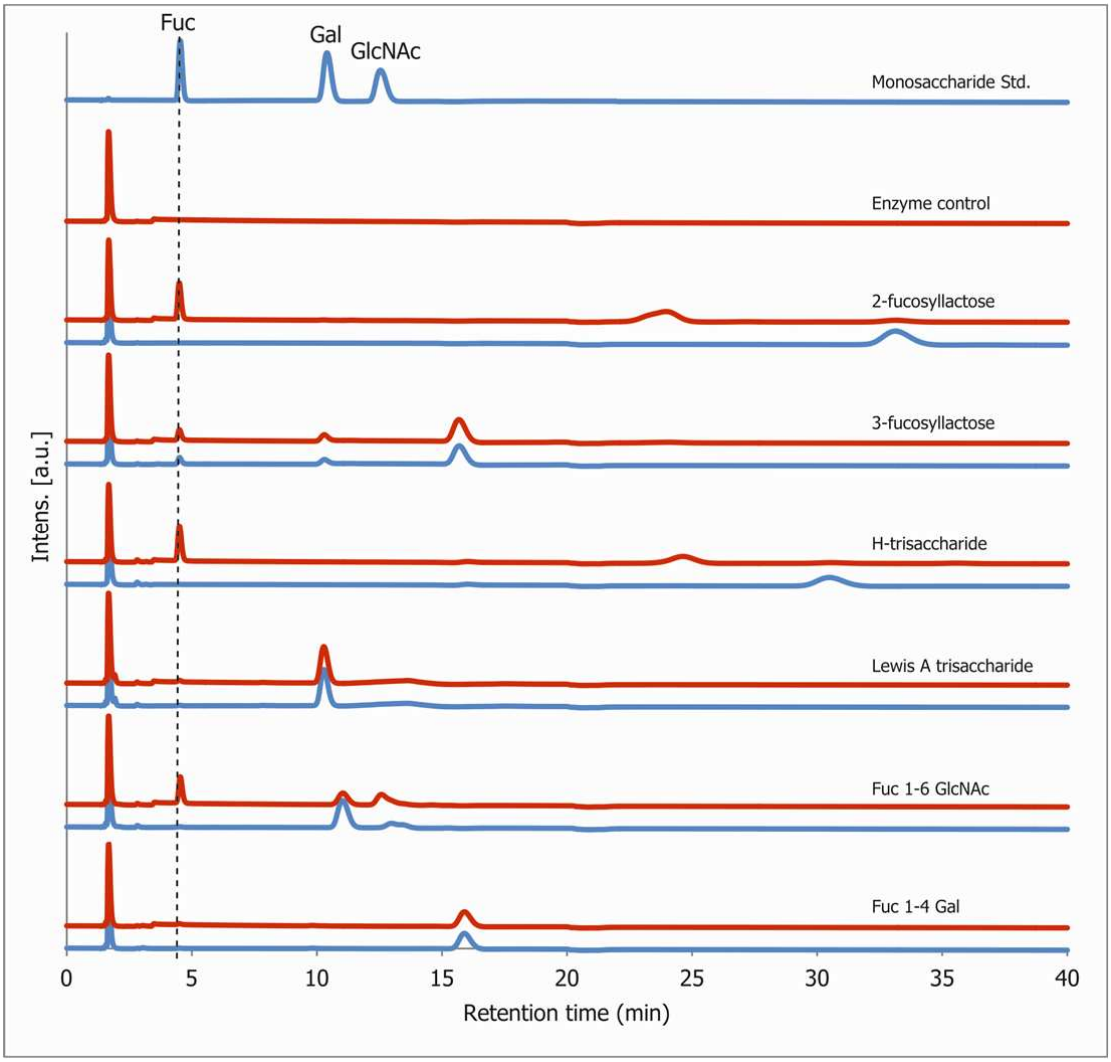


Figure 4. of Megson *et al.*

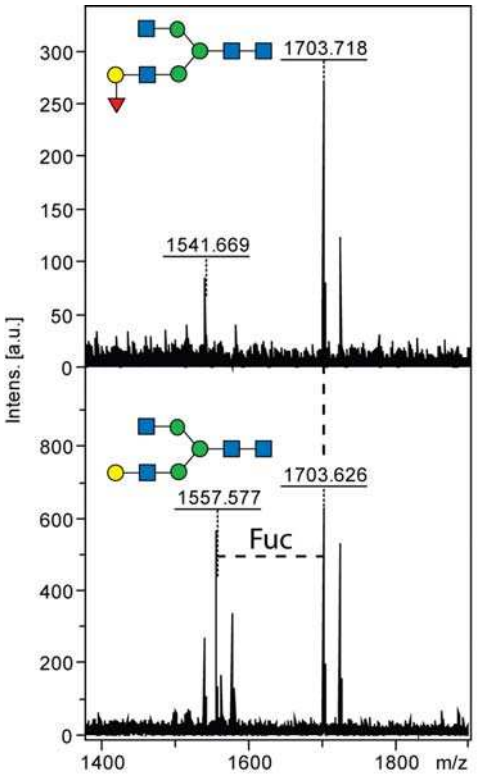


Figure 5. of Megson *et al.*

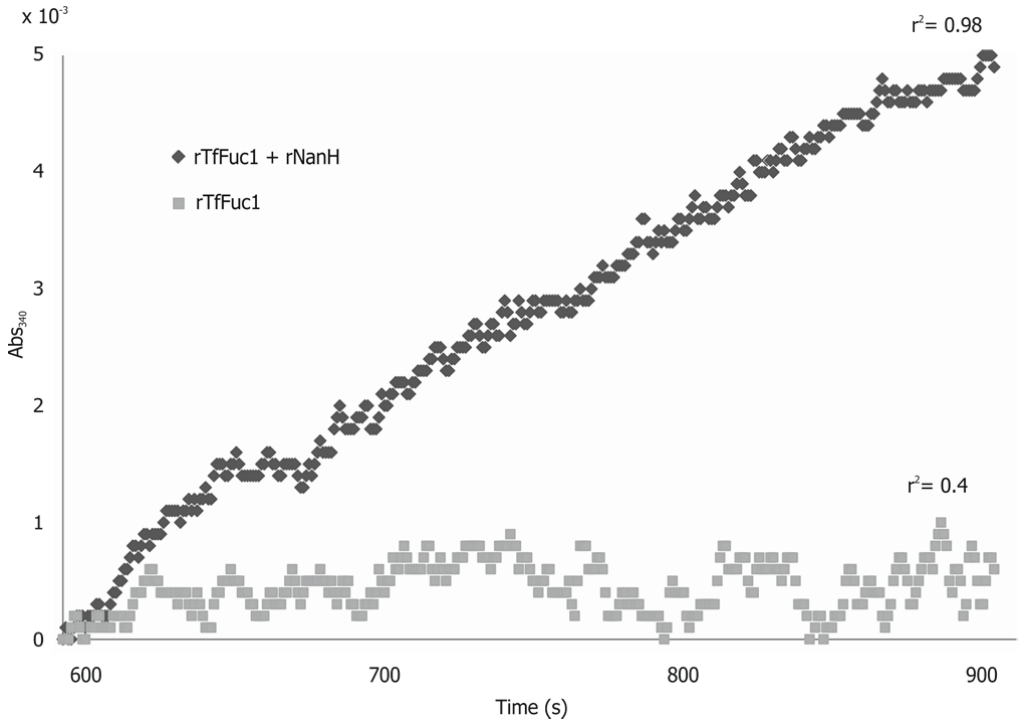
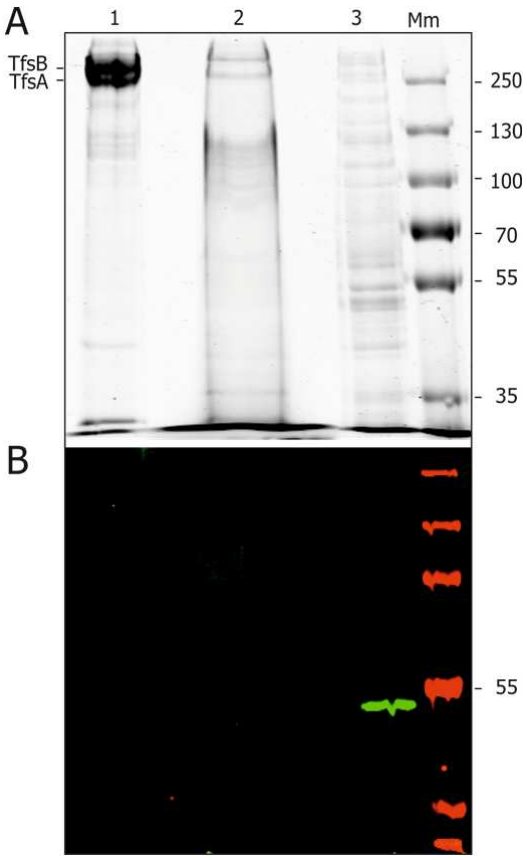


Figure 6. of Megson *et al.*



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Lewis A trisaccharide	β-D-Gal-1,3(α-L-Fuc-1,4)-β-D-GlcNAc	α(1,4)	No	-
Fuc(1,6)GlcNAc	α-L-Fuc-1,6-β-D-GlcNAc	α(1,6)	Yes	0.35
Fuc(1,4)Gal	α-L-Fuc-1,4-β-D-Gal	α(1,4)	No	-
A antigen tetraose	β-GalNAc-1,3(α-L-Fuc-1,2)-β-D-Gal-1,4-D-Glc	α(1,2)	No	-

*μmol/min/mg of enzyme

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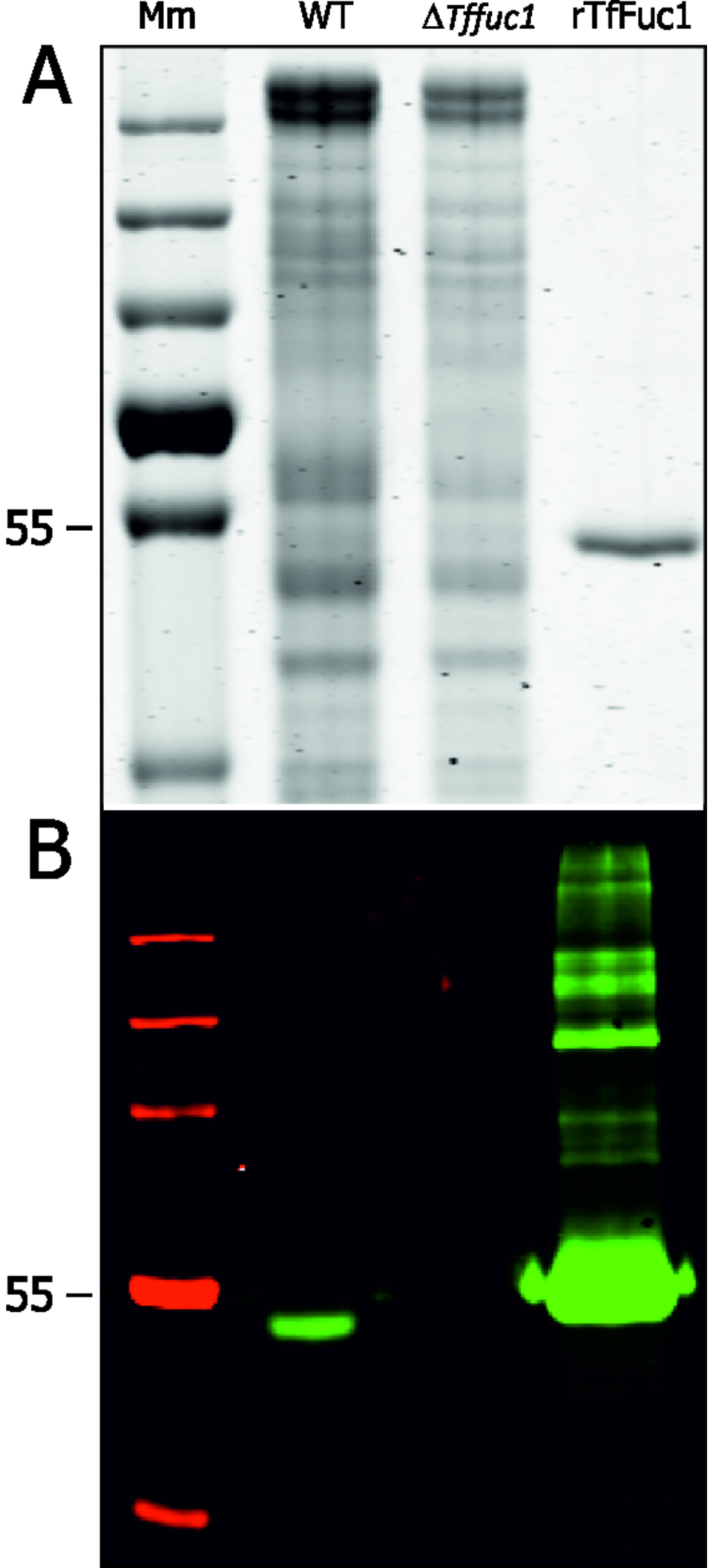
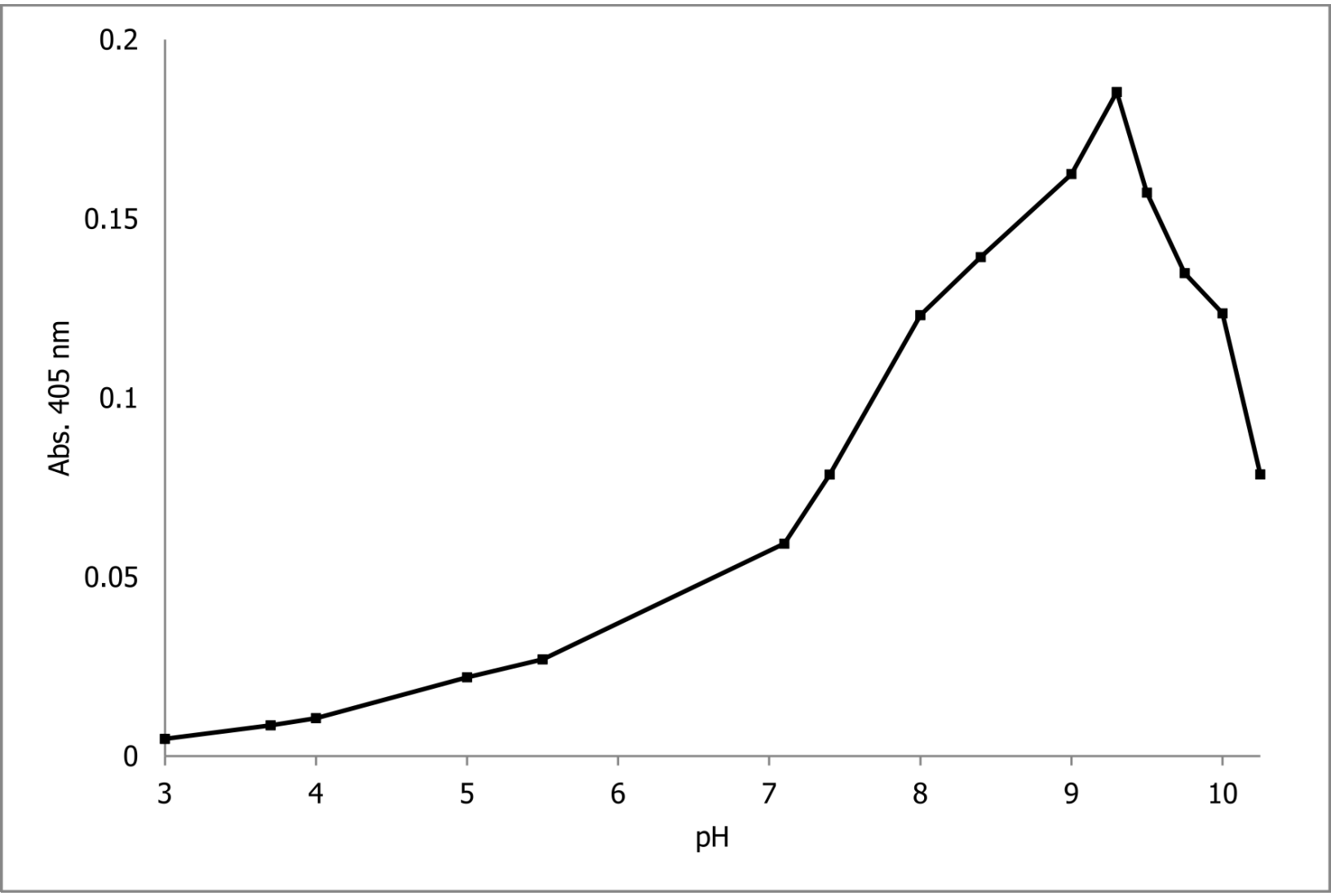
Escherichia coli BL21 (DE)	*F*$^-$*, ompT, hsdS* (*rB*$^-$*mB*$^-$), *gal, dcm* (DE3)	Invitrogen
Tannerella forsythia ATCC 43037	Wild-type isolate	American Type Culture Collection, USA
T. forsythia Δ*Tffuc1*	*T. forsythia* knockout of the *Tffuc1* gene; Erm^r	This study
pET-22b (+)	Expression vector with a His_6-tag, Amp^r	Novagen
pJET1.2-*Tffuc1*_ko	pJET1.2 carrying the *Tffuc1_ermF-AM* knockout cassette	This study

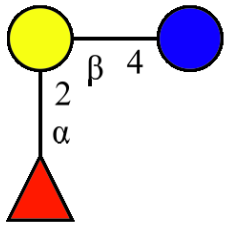
Table 4. Oligonucleotide primers used for PCR amplification reactions[a]

Primers	Sequence (5' → 3')[a]
1	gcggCATATGAAAACAAGAACATTACTTCTTTGTG
2	gctaCTCGAGGTTTAGAGGCAATTCATTGGCAAATG
3	GACCAAGCTGCAGGCCATCATCGATGTGCTCAAC
4	*GAAGCTATCGGGGGTACCTCCCCCGGG*-AGAATAATTTTTGTTTATTACTAAAAAATAACG
5	*GCTTCGGGGATCCTCTAGCCCCCGGG*- CAGAAATATCTTTATGAAACATCCTATTGATGGGGTG
6	GCTCAGCCAGCCGATAGTTACTTTTTTCGTTATGTGTTCCC
7[b]	**CGTTATTTTTTAGTAATAAACAAAAATTATTCT-**CCCGGGGGAGGTACCCCCGATAGCTTC
8[b]	**CACCCCATCAATAGGATGTTTCATAAAGATATTTCTG-**CCCGGGGGCTAGAGGATCCCCGAAGC
9	CACGATGAACGTGTCGGTCATTAAC
10	GAAGCTATCGGGGGTACCTCCCCCGGG
11	GCTTCGGGGATCCTCTAGCCCCCGGG
12	GCACATATTTAGTAACCCGATAGCC

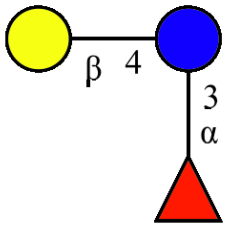
a. Artificial restriction sites are underlined. Lowercase letters indicate artificially introduced bases to improve restriction enzyme cutting.
In italics are the overlap sequences complementary to *ermF-ermAM*. In bold are the overlap sequences complementary to the *BFO_2737* flanking regions.
b. Primer sequences were taken from Honma et al.[11]


Mm
WT
ΔTffuc1
rTfFuc1
A
55
B
55


0.2
0.15
0.1
0.05
0
Abs. 405 nm
3
4
5
6
7
8
9
10
pH



2-fucosyllactose



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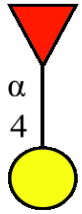
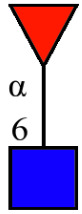
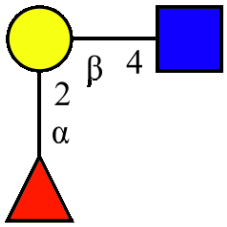


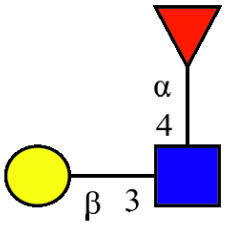
Fuc 1-4 Gal



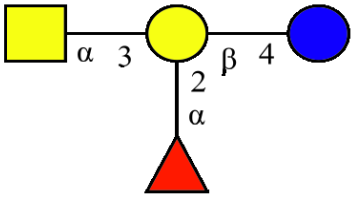
Fuc 1-6 GlcNAc



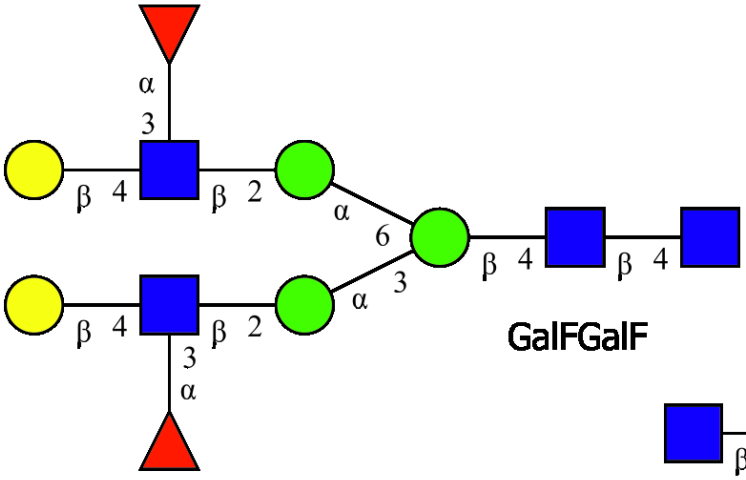
H-trisaccharide type I



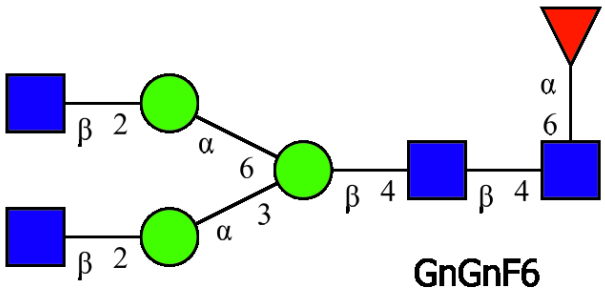
Lewis A trisaccharide



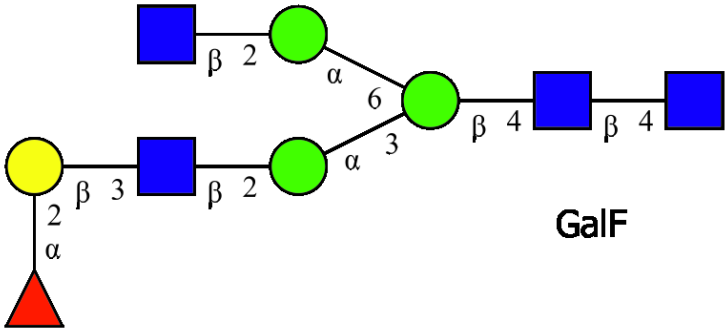
A antigen tetraose type 5



GalFGalF



GnGnF6



GalF

galactose

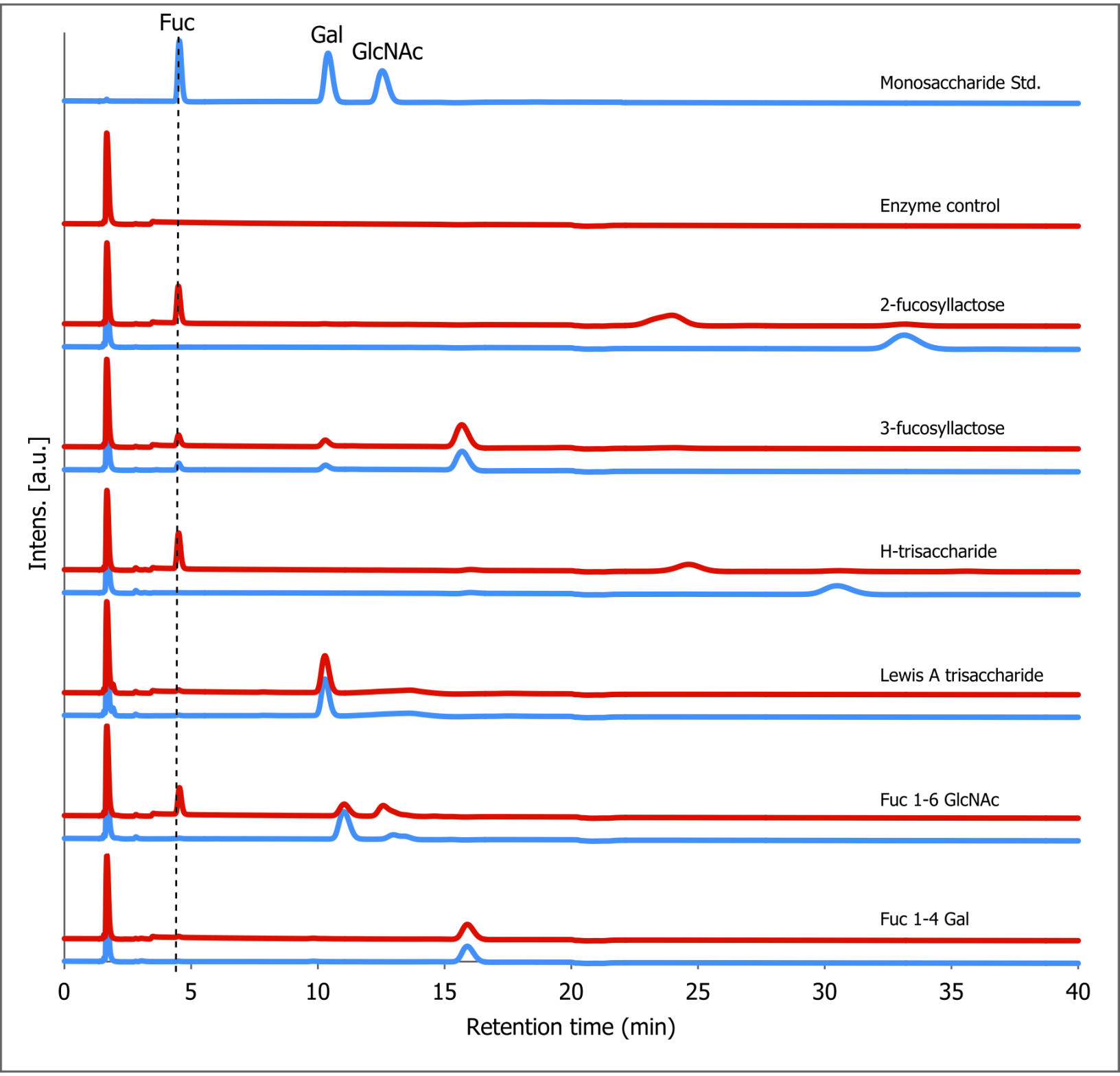
glucose

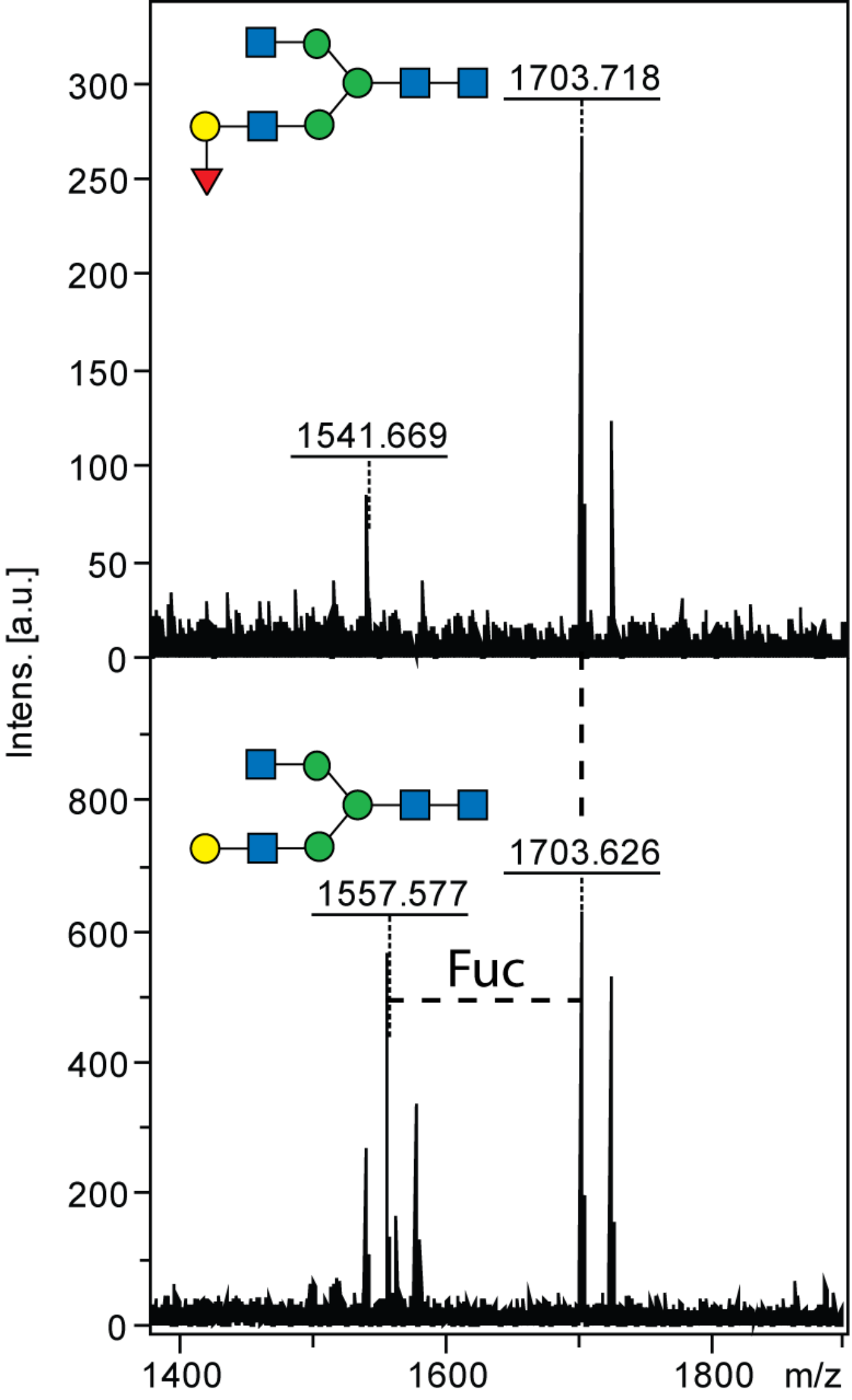
N-acetylgalactosamine

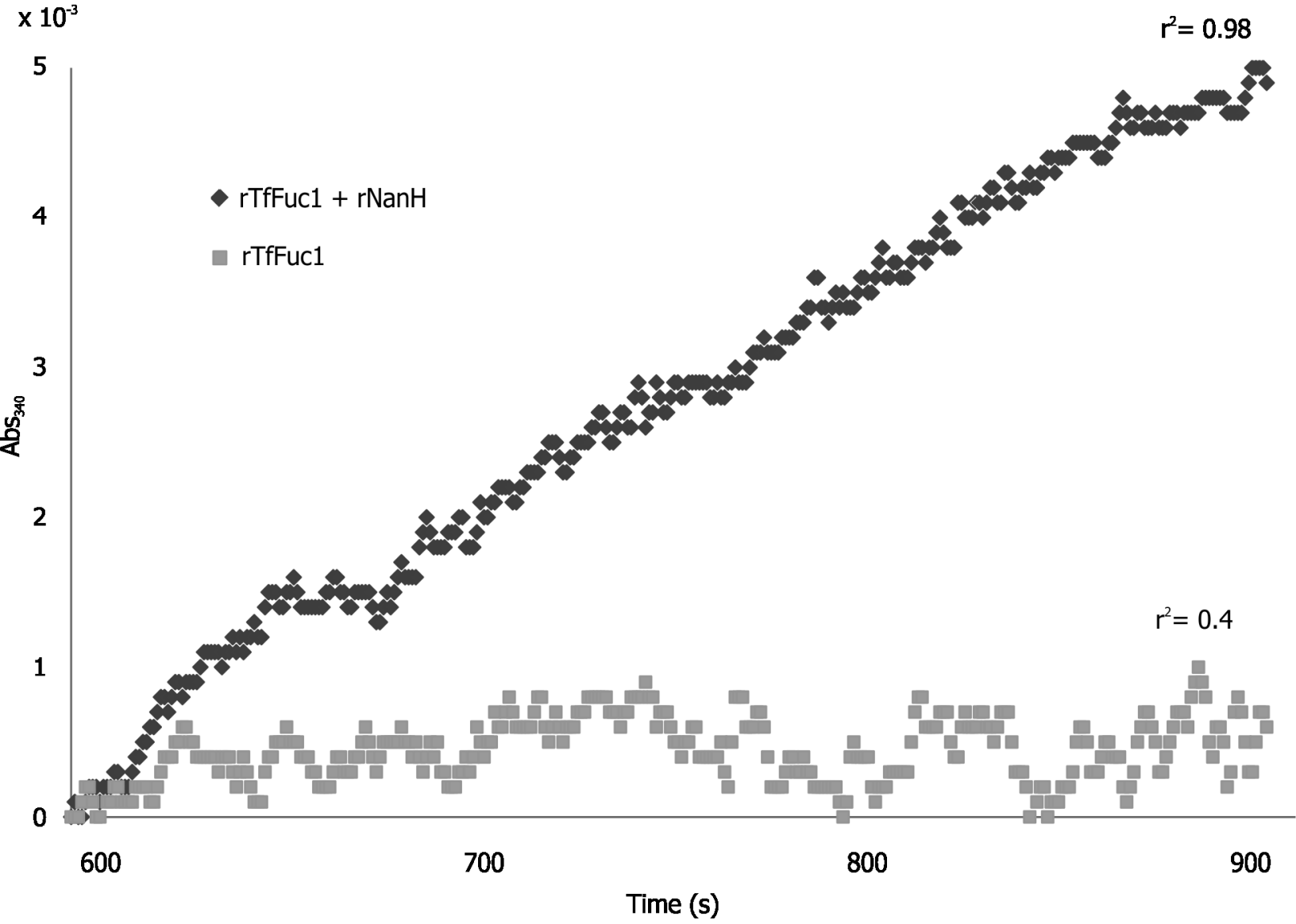
N-acetylglucosamine

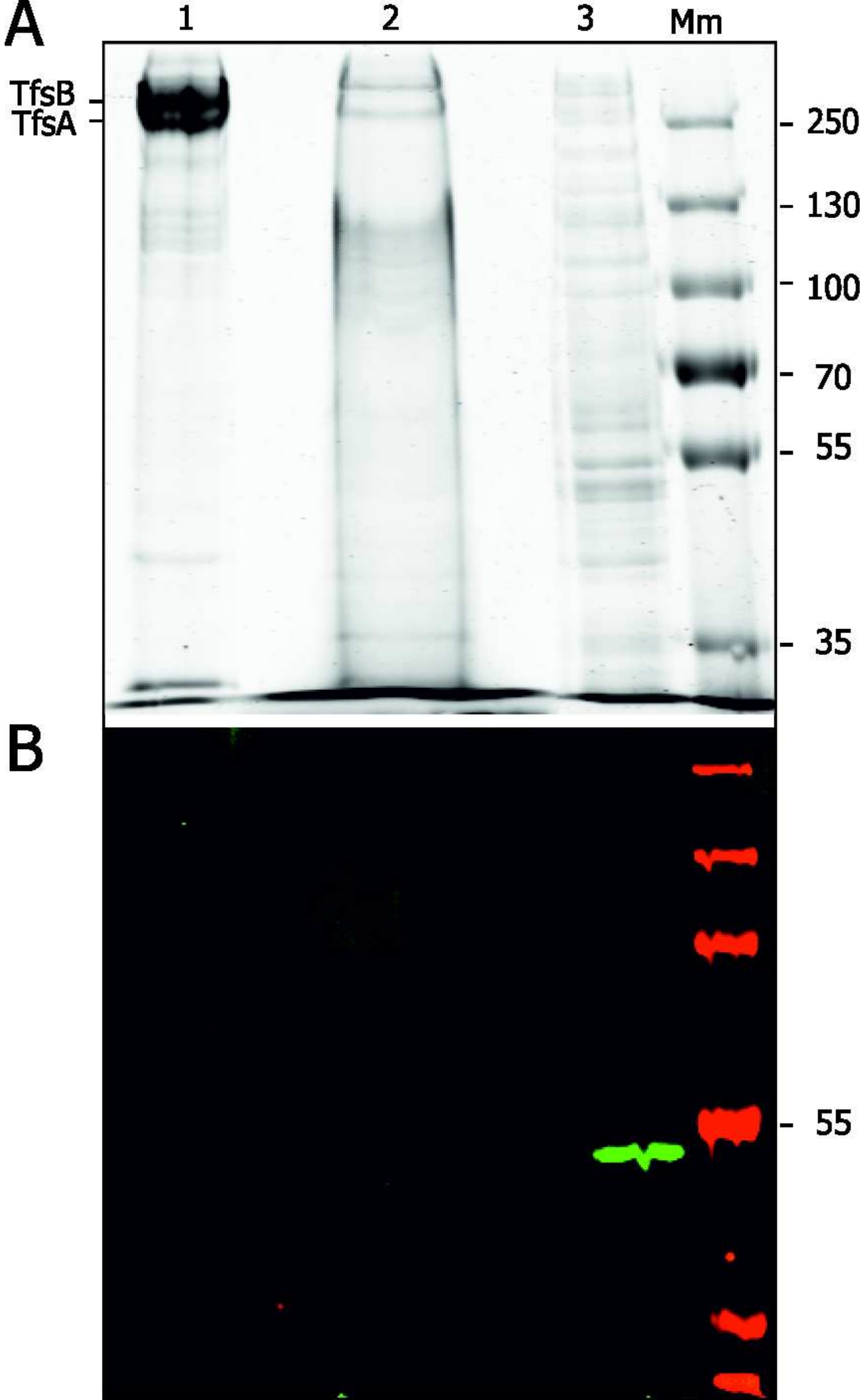
fucose

mannose


Fuc
Gal
GlcNAc
Monosaccharide Std.
Enzyme control
2-fucosyllactose
3-fucosyllactose
H-trisaccharide
Lewis A trisaccharide
Fuc 1-6 GlcNAc
Fuc 1-4 Gal
Intens. [a.u.]
0
5
10
15
20
25
30
35
40
Retention time (min)


1703.718
1541.669
1703.626
1557.577
Fuc
Intens. [a.u.]
300
250
200
150
100
50
0
800
600
400
200
0
1400
1600
1800
m/z


x 10-3
r2= 0.98
5
4
3
2
1
0
rTfFuc1 + rNanH
rTfFuc1
Abs340
r2= 0.4
600
700
800
900
Time (s)


A
1
2
3
Mm
TfsB
TfsA
250
130
100
70
55
35
B
55

Supplementary Information

Experimental Procedures

Attachment and invasion assays. The H357 oral epithelial cell line (CCL17; American Type Culture Collection) was maintained in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum and L-glutamine. The cultures were incubated at 37°C under 5% CO_2. H357 cells were grown to near-confluence (90 to 95%) for the assays.

For both attachment and invasion assays, T. forsythia WT and ΔTffuc1 strains were incubated with H357 cells at a multiplicity of infection of 100, as described previously.[1] Briefly, for attachment assays, epithelial cell monolayers incubated with bacteria for 1.5 h were washed three times with sterile PBS, epithelial cell-associated bacteria were retrieved by lysing monolayers by addition of distilled water before physical disruption by scraping, and bacteria were plated for counting. For invasion assays, epithelial cell monolayers incubated with bacteria were washed with DMEM and then treated with metronidazole (200 µg/ml) for 1 h to kill external bacteria. The monolayers were then washed with PBS and were lysed as above to allow intracellular bacteria counting.

Microtiter assays of Hoechst-stained biofilms. Tannerella forsythia WT and ΔTffuc1 biofilms were grown essentially as previously described in both full-strength and half-strength liquid BHI medium.[2] Before starting the bacterial cell culture, a 0.5-mg/ml solution of mucin from bovine submaxillary gland (Sigma) was prepared in 0.1 M sodium citrate buffer, pH 4.5, containing 0.1 M NaCl, added to non-treated polystyrene 24 well-plates (500 µl/well; Thermo Scientific) and incubated overnight at 37°C. On the next day, the mucin solution was removed completely and strains were inoculated at an OD_{600} ~0.05 in full-strength BHI medium supplemented with horse serum and N-acetylmuramic acid or in medium diluted 1:2 with

phosphate-buffered saline (PBS), yielding half-strength medium. In both cases, the medium was also supplemented with gentamycin (50 µg/ml) and gentamycin/erythromycin (50 µg/ml, 10 µg/ml) for WT and ΔTffuc1, respectively. Triplicates were made for each condition and a fourth well served for measurement of total growth (OD_{600}). The plates were incubated anaerobically for 48 h at 37°C; subsequently, the supernatant was removed and the biofilm was washed once with 800 µl PBS before adding 1 ml/well of 10 µg/ml Hoechst 33258 Fluorescent Stain (Thermo Scientific) in PBS.[3] The biofilm was then removed from the bottom of the well using vigorous pipetting and the plates were left in the dark on a shaker for 45 min. 100 µl from each well was placed in a black 96-well-plate with a flat optical bottom (Thermo Scientific) for fluorescence intensity readings using excitation/emission filters of 360(35)/485(20) in an Infinite 200 plate reader (TECAN). The fluorescence intensity was corrected by the total OD_{600} reached by each strain.

Immunofluorescence microscopy of T. forsythia cells using TfFuc1-specific polyclonal antiserum. The surface localization of TfFuc1 was investigated by immunofluorescence staining of T. forsythia WT as described elsewhere.[4]

Cells were grown until the late-exponential phase (OD_{600} ~1.0), washed with PBS and adsorbed on a glass slide for 2 h. All procedures were performed at 22°C, if not stated otherwise. Cells were washed twice with PBS and fixed for 30 min at -20°C in 70% (v/v) ethanol in PBS. After two washing steps with PBS, cells were incubated in blocking buffer [10%, (w/v) bovine serum albumin (Sigma) in PBS] for 1 h, followed by incubation in blocking buffer containing TfFuc1-specific polyclonal antiserum or pre-immune serum (1:10-dilution) for 1 h, washed once with blocking buffer and twice with PBS, and subsequently incubated in blocking buffer containing goat anti-mouse IgG (1:100) conjugated to FITC (Sigma-Aldrich) for 2 h. The cells were washed once with blocking buffer and twice with PBS to remove unbound antibodies. One drop of 50% (v/v) glycerol in PBS was added onto

the cells and a coverslip was mounted. Confocal laser scanning microscopy analysis was performed using a Leica TCS SP5 II system. Images were taken with a 63.0 x 1.40 oil-immersion objective. Immunofluorescence-stained bacterial cells were excited at 488 nm using an argon laser and detected at an emission bandwidth of 500-595 nm. Images were acquired and processed with Leica LAS AF software.

Results and Discussion

Attachment and invasion assays. The effect of the Tffuc1 mutation on the ability of T. forsythia to interact with and invade human oral epithelial cells was tested using an antibiotic protection assay on the oral epithelial cell line H357. Results are shown as percentage of the bacterial cells recovered compared to a viability control run in parallel during the course of the whole experiment.

The data shows no significant difference in invasion between WT and *Δ*fuc strains (**Fig. S1**). In addition, we compared the ability of the strains to attach to the epithelial cell line and again, no significant difference could be highlighted between the two (not shown), thus, indicating that TfFuc1 most likely does not play a role in direct human-pathogen interactions.

Microtiter assays of Hoechst-stained biofilms. T. forsythia WT and the ΔTffuc1 mutant were cultured anaerobically for 2 d in full-strength and half-strength medium in 24-well microtiter plates coated with mucin,[2] which is needed to promote biofilm formation as the presence of terminal sialic acid is an important factor allowing for T. forsythia surface attachment and biofilm growth.[5] Biofilm formation was evaluated by measuring the fluorescence intensity of the Hoechst-stained biofilm (**Fig. S2A**). As the different strains presented a different growth in the different conditions tested, the fluorescence intensity was corrected by the total OD_{600} reached in each experiment.

The WT strain increased its biofilm formation 1.4-fold as the strength of the medium was decreased to half by diluting with PBS. This indicates that biofilm formation is quite sensitive to changes of the medium strength, potentially indicating a degree of starvation (**Fig. S2A**).

In all experiments, the ΔTffuc1 had a higher biofilm formation phenotype than the WT strain, but how large the difference was depended largely on the strength of the growth medium. In full-strength BHI medium, the increased biofilm formation for the Δfuc mutant was 1.2-fold that of the WT. In the case of half-strength medium, the difference became more prominent as Δfuc presented a 1.5-fold increase (**Fig. S2B**). These results could indirectly indicate a difference between the strains in their ability to utilize and scavenge components in the medium but it is currently not clear whether the observed phenotype reflects solely the loss of Tffuc1 as no gene complementation assay was performed. We, therefore, conclude that the loss of the gene Tffuc1 does not lead to a decreased biofilm formation indicating no direct role of the protein in the interaction of T. forsythia with the mucin-coated surface.

References

1. Stafford P, Higham J, Pinnock A, Murdoch C, Douglas CW, Stafford GP, et al. Gingipain-dependent degradation of mammalian target of rapamycin pathway proteins by the periodontal pathogen Porphyromonas gingivalis during invasion. Mol Oral Microbiol 2013; 28:366-78.
2. Honma K, Mishima E, Inagaki S, Sharma A. The OxyR homologue in Tannerella forsythia regulates expression of oxidative stress responses and biofilm formation. Microbiology 2009; 155:1912-22.
3. Tomek MB, Neumann L, Nimeth I, Koerdt A, Andesner P, Messner P, et al. The S-layer proteins of Tannerella forsythia are secreted via a type IX secretion system that is decoupled from protein O-glycosylation. Mol Oral Microbiol 2014; 29:307-20.

4. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York 1989.
5. Roy S, Honma K, Douglas CW, Sharma A, Stafford GP. Role of sialidase in glycoprotein utilization by Tannerella forsythia. Microbiology 2011; 157:31953-202.

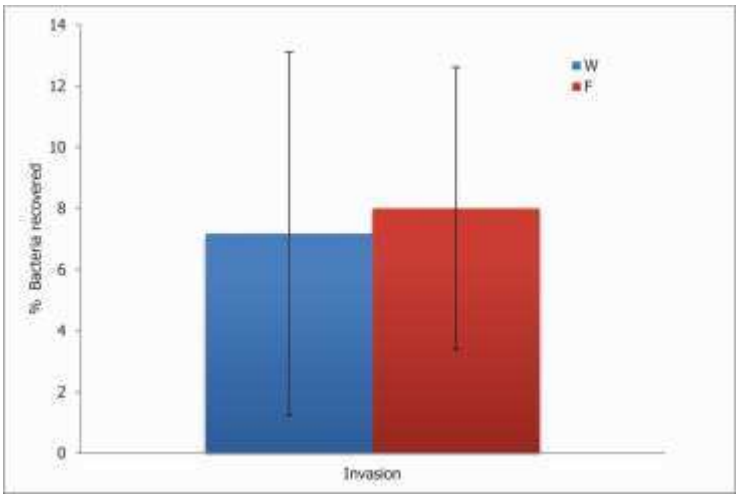


Figure S1. Cell invasion assays. The effect of the Tffuc1 mutation on the ability of T. forsythia to interact and invade human oral epithelial cells was tested on the oral epithelial cell line H357. The combined results from three experimental repeats are shown as a percentage of the bacterial cells recovered compared to a viability control. W, T. forsythia wild-type; F, T. forsythia ΔTffuc1.

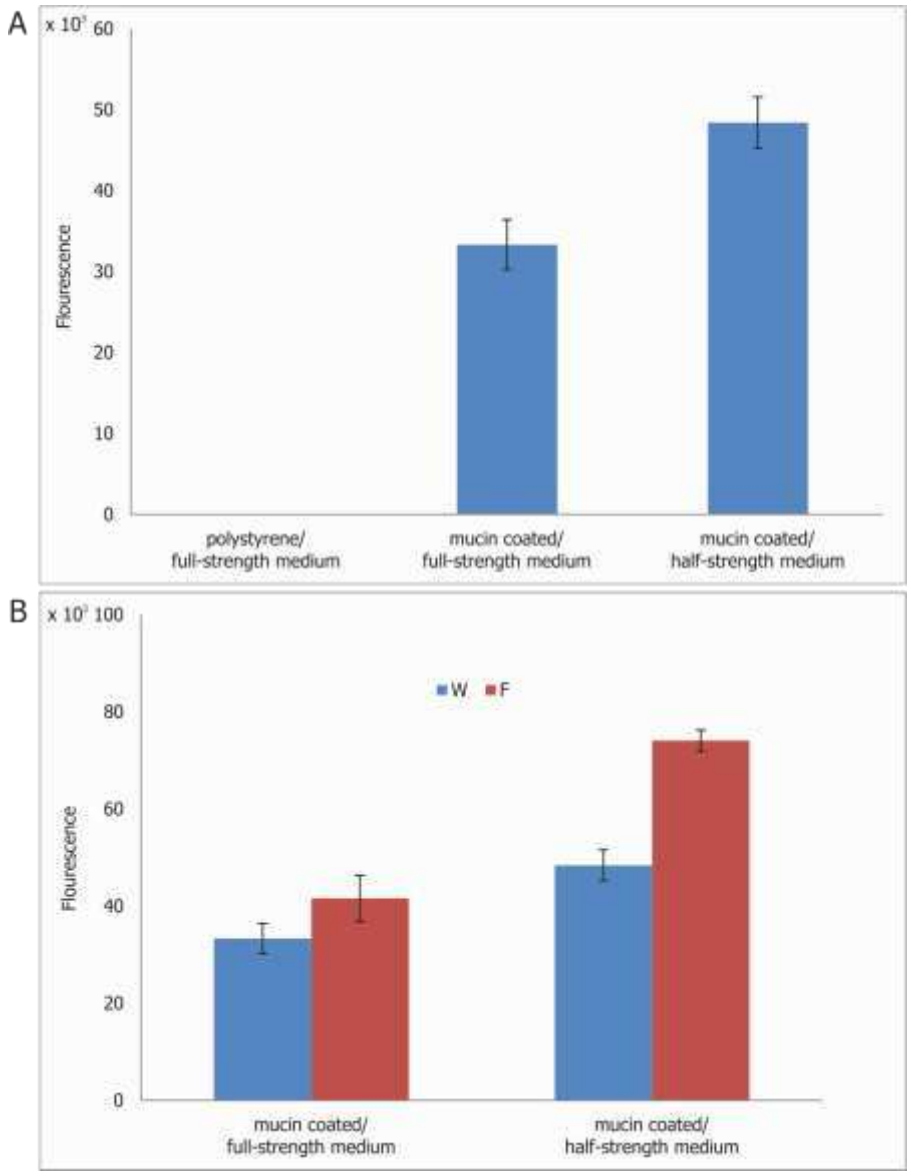
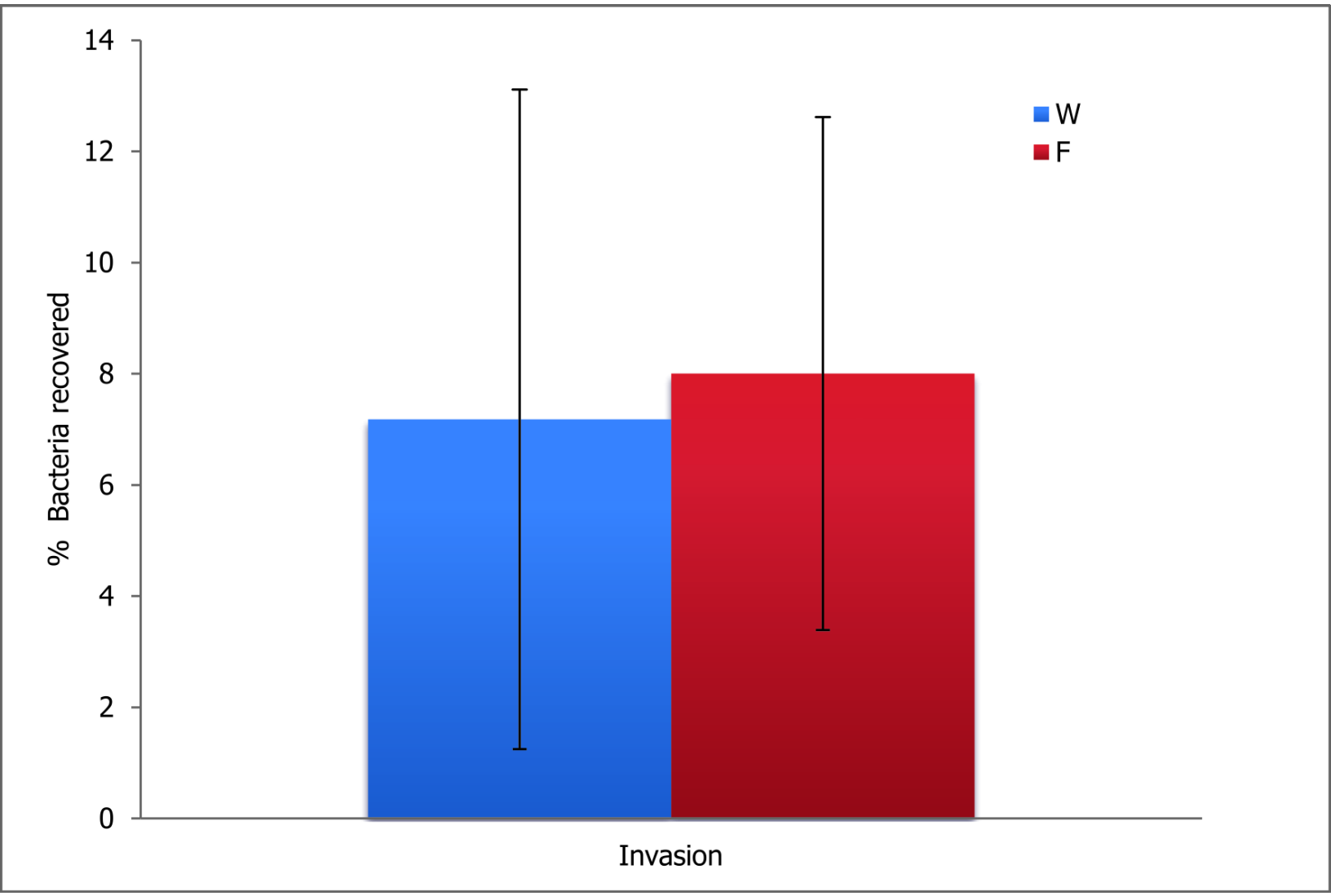
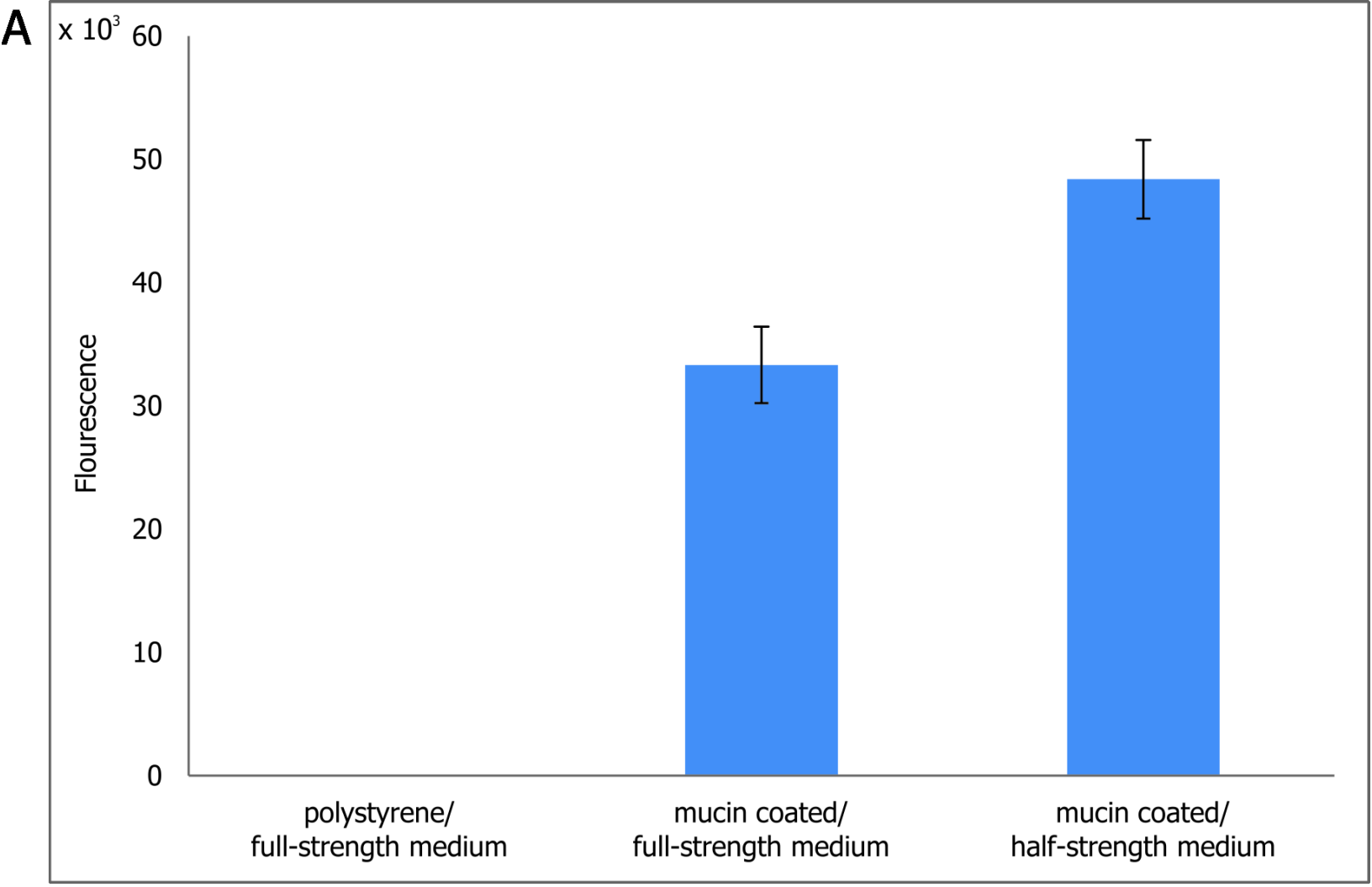
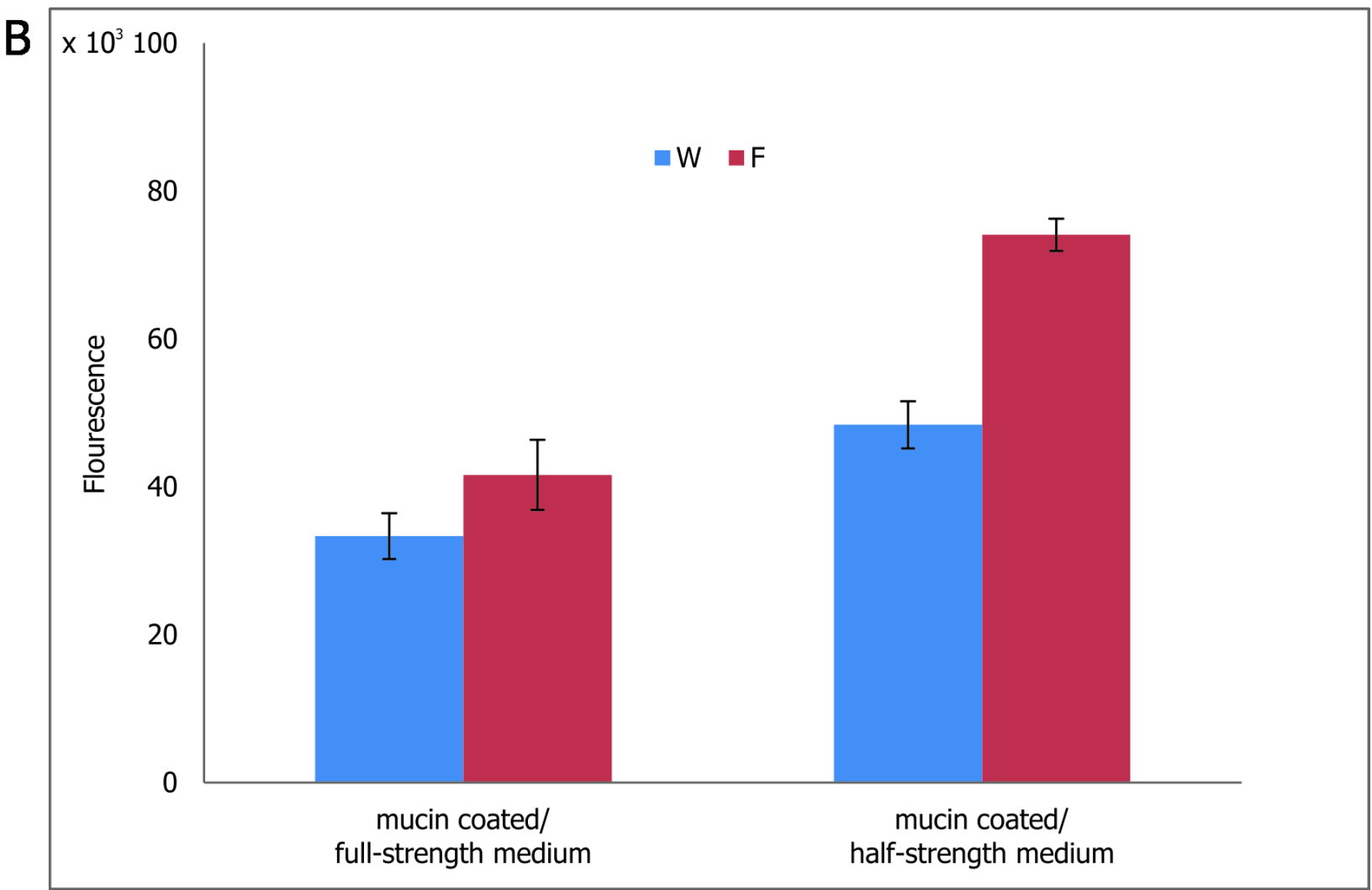


Figure S2. Microtiter assays of Hoechst-stained biofilms. **A.** The WT strain increased its biofilm formation on a mucin-coated surface as the strength of the medium was decreased to half that of the original broth. **B.** The difference in biofilm formation between the WT and the ΔTffuc1 depends largely on the strength of the medium. W, T. forsythia wild-type; F, T. forsythia ΔTffuc1.


14
12
10
8
6
4
2
0
% Bacteria recovered
W
F
Invasion


A
x 10^3
60
50
40
30
20
10
0
Flourescence
polystyrene/
full-strength medium
mucin coated/
full-strength medium
mucin coated/
half-strength medium


B
x 10^3
100
80
60
40
20
0
Flourescence
W
F
mucin coated/
full-strength medium
mucin coated/
half-strength medium