



This is a repository copy of *Characterization of an alpha-L-fucosidase from the periodontal pathogen Tannerella forsythia*.

White Rose Research Online URL for this paper:
<http://eprints.whiterose.ac.uk/90057/>

Version: Accepted Version

Article:

Megson, Z.A., Koerdt, A., Schuster, H. et al. (8 more authors) (2015) Characterization of an alpha-L-fucosidase from the periodontal pathogen *Tannerella forsythia*. *Virulence*, 6 (3). 282 - 292. ISSN 2150-5594

<https://doi.org/10.1080/21505594.2015.1010982>

Reuse

Unless indicated otherwise, fulltext items are protected by copyright with all rights reserved. The copyright exception in section 29 of the Copyright, Designs and Patents Act 1988 allows the making of a single copy solely for the purpose of non-commercial research or private study within the limits of fair dealing. The publisher or other rights-holder may allow further reproduction and re-use of this version - refer to the White Rose Research Online record for this item. Where records identify the publisher as the copyright holder, users can verify any specific terms of use on the publisher's website.

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

1 *Submitted to Virulence*

2
3 *Research Paper*

4
5 **Characterization of an α -L-fucosidase from the periodontal**
6 **pathogen *Tannerella forsythia***

7
8 Z. A. Megson¹, A. Koerdts¹, H. Schuster¹, R. Ludwig², B. Janesch¹, A. Frey³,
9 K. Naylor³, I. B.H. Wilson⁴, G. P. Stafford³, P. Messner¹ and C. Schäffer^{1*}

10
11 ¹Department of NanoBiotechnology, *NanoGlycobiology* unit, Universität für Bodenkultur
12 Wien, Vienna, Austria

13 ²Department of Food Science and Technology, Universität für Bodenkultur Wien, Vienna,
14 Austria

15 ³Integrated BioSciences, School of Clinical Dentistry, University of Sheffield, Sheffield, UK

16 ⁴Department of Chemistry, Universität für Bodenkultur Wien, Vienna, Austria

17
18
19 *Correspondence to: Christina Schäffer; E-mail: christina.schaeffer@boku.ac.at

1 **Abstract**

2

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

22

1 Introduction

2

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

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

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

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

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

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

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

3 Here, we describe the molecular cloning and characterization of BFO_2737, which we
4 named Tffuc1. This protein has previously been reported to form part of an outer membrane
5 preparation of *T. forsythia*²⁴ and, thus, was a good candidate to be involved in host-pathogen
6 interactions. Tffuc1 is a 446-amino acid protein with a theoretical pI and molecular mass of
7 6.9 and 50.8 kDa, respectively. It is the first fucosidase in this organism to be characterized to
8 date. The enzyme was shown here to be an α (1,2)-fucosidase and also possesses an α (1,6)
9 specificity on small unbranched substrates. It is a predicted periplasmic protein, possibly
10 playing a role in the breakdown of small oligosaccharides. It is, to the best of our knowledge,
11 the first glycosyl hydrolase in its family (GH29) reported to be a specific α (1,2)-fucosidase.

13 Results

14
15 **Enzymatic characterization of rTffuc1.** The *Tffuc1* gene was cloned into pET22-b(+)
16 vector and expressed in *E. coli* as a C-terminally His₆-tagged protein, which enabled
17 purification via nickel affinity chromatography (**Fig. 1**). The enzymatic activity was then
18 tested using the standard colorimetric α -fucosidase substrate 4-nitrophenyl- α -L-
19 fucopyranoside (pNP-fucose) at 22°C in a range of different pH values and in the presence of
20 MgCl₂, KCl and NaCl, in order to establish its pH optimum and cation dependence,
21 respectively. By stopping the reaction with the addition of an alkaline buffer at pH 11.4, it
22 was ensured that all wells were at the same pH for consequent absorbance readings. The
23 activity of the enzyme was seen to start to plateau at the neutral to alkaline pH range and was
24 considered most active at pH 9.0, assayed in glycine buffer, and not at pH 9.25 where the
25 activity suddenly peaks and then rapidly decreases thereafter. The activity remained largely
26 unaffected by the presence of cations at the two concentrations tested (results not shown). The

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

6
7 **Substrate linkage specificity of rTffuc1.** To determine the enzyme linkage specificity,
8 rTffuc1 was incubated with a set of different fucosylated substrates of defined structure
9 representing a range of fucose linkages available on host glycoproteins and on oral surfaces
10 (**Fig. 3**). The reaction products obtained after overnight incubation were analysed using high-
11 pH anion-exchange chromatography with pulsed amperometric detection (HPAEC) where the
12 release of fucose was confirmed by comparison with the retention time of the standard
13 monosaccharide and of a substrate standard after overnight incubation at 37°C. The enzyme
14 was seen to be active on both $\alpha(1,2)$ fucose containing substrates, 2-fucosyllactose and H-
15 trisaccharide, and on the $\alpha(1,6)$ fucose disaccharide α -L-Fuc-(1,6)- β -D-GlcNAc, although this
16 latter reaction did not reach completion, indicating weak specificity for this linkage. The
17 $\alpha(1,3)$ and $\alpha(1,4)$ linkages were not cleaved on 3-fucosyllactose and the Lewis A
18 trisaccharide, respectively. The enzyme was also inactive on the substrate α -L-Fuc-(1,4)- β -D-
19 Gal, added as a second disaccharide control to prove that the $\alpha(1,6)$ activity was not due to
20 differences in substrate length (**Fig. 4**).

21 In order to obtain accurate activity values on the cleaved substrates, the K-FUCOSE
22 kit from Megazyme was used, coupled to the enzymatic reaction with rTffuc1. First, FDH,
23 which also has an alkaline pH optimum, and $NADP^+$ were added to the substrate solution
24 reaction mixture in order to convert any free fucose already present in the sample to L-fucono-
25 1,5-lactone by the reduction of $NADP^+$ to NADPH ($\epsilon_{340} = 6.022 \text{ mM}^{-1} \text{ cm}^{-1}$). rTffuc1 was then
26 added to the mixture and the reaction was monitored by following the increase in Abs_{340} . The

1 activity was calculated from where the formation of NADPH was linear over time. The
2 enzyme was most active on 2-fucosyllactose and H-trisaccharide with specific activities of
3 0.8 U/mg and 0.6 U/mg, respectively. The activity on the $\alpha(1,6)$ disaccharide was
4 significantly lower at 0.35 U/mg (**Table 2**).

5 The activity of the enzyme on the various substrates could be calculated
6 approximately (as some loss of material occurred during sample preparation) from the HPLC
7 experiments after 1-h incubation periods (results not shown) and was found to be markedly
8 lower than that observed with the K-FUCOSE kit, indicating that free fucose, which is
9 consumed in the latter, could be inhibiting the enzymatic activity significantly. In order to
10 determine the extent of such an effect, measurement of K_M and V_{max} values were repeated
11 with pNP-fucose in the presence of either 0.25 mM L-fucose or 0.1 μ M deoxyfuconojirimycin
12 (DFJ), which is a strong fucosidase inhibitor.²⁸ The enzyme was competitively inhibited by
13 both fucose and DFJ as the V_{max} remained largely unaffected but the K_M value increased from
14 0.67 mM to 16.5 mM and 28.3 mM, respectively (**Table 1**).

15 Further, the ability of the enzyme to cleave fucose residues off more complex
16 natural glycans and those on branched sugar residues was assayed (compare with **Fig. 3**). As
17 expected, the enzyme was unable to cleave the $\alpha(1,3)$ fucose linkage on GalFGalF-pep,
18 included in the assays as a trace amount of activity could be observed when using 3-
19 fucosyllactose, as measured by the K-FUCOSE kit. The enzyme was also not able to cleave
20 the core α -1,6 fucose linkage on GnGnF⁶-pep nor the branched $\alpha(1,2)$ -linked fucose on the
21 A antigen. The non-branched $\alpha(1,2)$ fucose linkage present on the Eastern oyster substrate,
22 however, was cleaved off the substrate GalF, seen by the loss of a fucose residue in the MS
23 spectra of the substrate. The major m/z 1703 glycan ($[M+H]^+$) was approximately 50%
24 digested to a defucosylated species of m/z 1557 after overnight incubation with the enzyme
25 (**Fig. 5**). The enzyme is, therefore, able to cleave off fucose residues which are $\alpha(1,2)$ linked
26 on more complex glycans only when in a terminal unbranched position and is unable to cleave

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

2

3 **rTfFuc1 activity on bovine submaxillary mucin.** rTfFuc1 was incubated with mucin from
4 bovine submaxillary glands and the release of fucose was measured with the K-FUCOSE kit.
5 Incubation was performed also in combination with rNanH from *T. forsythia* and activities
6 were calculated from the slope of ΔAbs_{340} where it was linear over time. During the assayed
7 incubation period of 10 min, no activity could be detected when rTfFuc1 was incubated alone
8 with mucin. The ΔAbs_{340} lead to an irregular data set with a very low r^2 value. However,
9 fucose release was detectable when the incubation was performed in conjunction with the
10 rNanH sialidase, presenting a slow but steady increase in the Abs_{340} . The activity was
11 calculated over a period of 300 s where the data points fitted a linear regression with an r^2 of
12 0.98 (**Fig. 6**). The experiment was repeated several times and yielded an activity of 24
13 ± 4 mU/mg. rTfFuc1 might cleave fucose off mucin over longer periods of time, but the data
14 shows a significantly higher activity when sialic acid residues are first removed from mucin,
15 indicating that the fucosidase TfFuc1 could work downstream from the sialidase in *T.*
16 *forsythia* and presumably cooperate with other glycosidases in the degradation of complex
17 glycans.

18

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

1 comprising both the cytoplasmic and periplasmic content (**Fig. 7**), arguing against surface
2 localization of the Tffuc1 enzyme.

3

4 **Discussion**

5

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

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

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

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

9 During the course of this study, it became clear that one of the issues possibly
10 underlining our observations was the enzyme's cellular localization. Even though TfFuc1 was
11 found previously to be present in the outer membrane proteome of the pathogen,²⁴ localization
12 of the enzyme on the surface of *T. forsythia* cells by fluorescent immunolabelling was not
13 successful (data not shown; see Experimental Procedures in the Supplementary Information)
14 and cell fractionation also showed the detectable protein to be found in the non-membrane
15 associated fraction (**Fig. 7**). This would be in agreement with bioinformatics interrogation
16 indicating that, while it has a Sec-dependent secretion signal (as predicted by SignalP 4.1
17 Server), it is not predicted to reside in the outer membrane or be secreted, nor does it contain a
18 T9SS C-terminal secretion signal.³² The difference in location of TfFuc1 between the present
19 study and the outer membrane proteomics experiment²⁴ can be reconciled when considering
20 that in the latter study, cross-contamination of individual proteins between cellular fractions
21 was not investigated.

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

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

5 This notion that the *T. forsythia* fucosidase TfFuc1 plays an internal role was
6 corroborated when we tested the effect of the $\Delta Tffuc1$ mutation on the ability of *T. forsythia* to
7 interact with and invade human oral epithelial cells using an antibiotic protection assay on the
8 oral epithelial cell line H357.³³ We found no significant differences in the ability of the
9 $\Delta Tffuc1$ to invade these human cells as compared to the WT strain (**Fig. S1**), indicating that
10 TfFuc1 has no effect on epithelial cell-invasion under the conditions tested. In addition, the
11 mutant did not show hindered biofilm formation when cultured on bovine submaxillary
12 mucin, contrary to the *T. forsythia NanH* sialidase mutant,¹⁵ but showed a slightly increased
13 biofilm formation (**Fig. S2**).

14 Our data supports the idea of a periplasmic fucosidase involved in the final
15 breakdown of small substrates that have been internalized, possibly owing to the action of
16 exoglycosidases and endoglycosidases which break-down larger glycans on the outside of the
17 cell.^{34, 35} TfFuc1 would possibly then be able to exert its full potential freeing both $\alpha(1,2)$ and
18 $\alpha(1,6)$ fucoses on small linear substrates. These findings are also in agreement with the
19 hypothesis that the fucosidase acts downstream of the sialidases, which have been shown to
20 act on whole glycoproteins on bacterial and host surfaces.^{14, 15, 36} The sialic acid would,
21 therefore, already be missing as smaller parts of the glycan are transported inside the cell.

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

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

6
7 Overall, our data suggest that Tffuc1 is a unique α -L-(1,2)-fucosidase which could potentially
8 contribute to fucose utilization in *T. forsythia*. In order to better elucidate this role and rule out
9 any redundancy in the system, two further annotated fucosidases in *T. forsythia*, BFO_1182
10 and BFO_3101, together with the annotated fucose permease, BFO_0307, are now being
11 investigated to elucidate the role of fucose in the physiology of *T. forsythia*.

12

13 **Experimental Procedures**

14

15 **Bacterial strains, medium and culture conditions.** *T. forsythia* wild-type (WT) strain
16 ATCC 43037 (American Type Culture Collection) and the knock-out mutant $\Delta Tffuc1$ were
17 grown anaerobically at 37°C for 4-7 d in brain heart infusion (BHI) broth or 0.8% (w/v) BHI
18 agar, supplemented with *N*-acetylmuramic acid (NAM), horse serum and gentamycin as
19 described previously.³² *Escherichia coli* DH5 α and BL21 (DE3) (Invitrogen) were cultivated
20 in selective Luria Bertani (LB) medium (agar and broth) supplemented with 100 μ g/ml
21 ampicillin (Amp). All strains and plasmids used in the course of this study are summarised in
22 **Table 3.**

23

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

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

12
13 **Recombinant production of His₆-tagged Tffuc1.** The *Tffuc1* gene was amplified from the
14 chromosome of *T. forsythia* ATCC 43037 with a fused C-terminal His₆-tag by PCR using
15 primer pair 1/2 (**Table 4**). The His₆-tagged amplification product was digested using
16 restriction enzymes NdeI/XhoI and cloned into NdeI/XhoI-linearized pET22-b (Novagen).
17 The corresponding plasmid was transformed into *E. coli* BL21 (DE3) cells for protein
18 expression. Freshly transformed cells were grown in two 400-ml Erlenmeyer flasks to an
19 OD₆₀₀ of 0.4-0.5 in the presence of 100 µg/ml of Amp at which point protein expression was
20 induced with a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and
21 cultures were shaken (200 rpm) overnight at 18°C. Cells were harvested by centrifugation
22 (6,500 g, 20 min, 4°C).

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

1 and the His₆-tagged protein was purified using an imidazole gradient in buffer A; 25 mM
2 imidazole (10 ml), 50 mM imidazole (10 ml), followed by five elution steps with 500 µl of
3 250 mM imidazole in buffer A. Eluted fractions containing the purified recombinant protein,
4 rTfFuc1, as determined by SDS-PAGE analysis, were pooled and dialysed overnight at 4°C
5 against 3 l of 10 mM phosphate buffer, pH 8.0. The volume was then reduced 5-fold using a
6 concentration centrifuge yielding a protein concentration of 0.35 mg/ml (as determined by
7 Nanodrop) in 50 mM phosphate buffer.

8

9 **Construction of a *T. forsythia* $\Delta Tffuc1$ knock-out strain.** Disruption of the *Tffuc1* gene in
10 *T. forsythia* was performed by gene knockout, as described previously.³² The *Tffuc1* gene is
11 not part of an operon, thus, downstream effects due to the chosen mutation strategy are not
12 expected to occur. Briefly, the flanking genomic regions (1,000 bp) up-stream and down-
13 stream of *Tffuc1* were amplified using primer pairs 3/4 and 5/6, respectively (**Table 4**). The
14 two resulting fragments were joined with the erythromycin resistance gene *ermF-ermAM*
15 (amplified using primer pair 7/8) by overlap extension PCR and sub-cloned into the blunt-end
16 cloning vector pJET1.2 (Thermo Scientific), resulting in pJET1.2/*Tffuc1*_ko. Approximately
17 5 µg of the knockout cassette was transferred by electroporation into 100 µl of competent *T.*
18 *forseythia* cells. Cells were regenerated in BHI medium for 24 h before plating on BHI agar
19 plates containing erythromycin (10 µg/ml) as a selection marker. Single colonies were picked
20 and used for inoculation of liquid BHI medium. Genomic DNA of the new $\Delta Tffuc1$ mutants
21 were isolated as mentioned above and the absence of the *Tffuc1* gene and the correct
22 integration of the erythromycin resistance gene (upstream and downstream) was evaluated by
23 PCR using primer pairs 1/2, 9/10, and 11/12, respectively (**Table 4**). Absence of the enzyme
24 in the $\Delta Tffuc1$ strain was also confirmed by Western immunoblotting of the total cell extract
25 separated by SDS-PAGE using TfFuc1-specific polyclonal antiserum (**Fig. 1**).

1 **General and analytical methods.** SDS-PAGE was carried out according to a standard
2 protocol using a Protean II electrophoresis apparatus (Bio-Rad).³⁹ Protein bands were
3 visualized with Coomassie Brilliant Blue G 250 (CBB) staining reagent. For Western
4 immunoblotting of proteins onto a nitrocellulose membrane (PeqLab), a Mini Trans-Blot Cell
5 (Bio-Rad) was used. Detection of the His₆-tag fused to rTfFuc1 and detection of TfFuc1 was
6 done with the Li-Cor Odyssey Infrared Imaging System using an anti-His₆ mouse antibody
7 (Roche) or TfFuc1-specific polyclonal antiserum raised in mice (EF-BIO), respectively, both
8 in combination with goat anti-mouse IgGIR Dye 800CW conjugate (Li-Cor).

9

10 **Enzymatic characterization of rTfFuc1.** Enzymatic characterization of rTfFuc1 was
11 performed essentially as described elsewhere.⁴⁰ 0.17 μ M of purified, recombinant enzyme
12 was incubated with 5 mM of the colorimetric substrate 4-nitrophenyl- α -L-fucopyranoside
13 (pNP-fucose) (Sigma) at a range of different pH values (3-10.25) and cation concentrations in
14 96-well plates at 22°C in a total volume of 40 μ l. The enzymatic reaction was stopped after
15 3 min by addition of 260 μ l of phosphate buffer, pH 11.4. Citrate/phosphate buffer (0.1 M)
16 was used to assay the pH range from 3-8,⁴¹ 50 mM glycine buffer was used for a pH range
17 from 8-10. The effect of MgCl₂ (5 mM, 10 mM), KCl (5 mM, 10 mM) and NaCl (50 mM,
18 150 mM) on the enzyme's activity was assayed in the same way in 50 mM glycine buffer,
19 pH 9.

20 A 4-nitrophenol standard curve was made by measuring the absorbance at 405 nm
21 (Abs₄₀₅) of 0, 4, 8, 12, 16, 20 and 24 nmol of 4-nitrophenol per well in 300 μ l of phosphate
22 buffer, pH 11.4. The K_M and V_{max} catalytic constants were calculated at pH 9.0 in
23 50 mM glycine buffer at 22°C in the presence of 0.01 to 50 mM pNP-fucose. The inhibitory
24 effect of fucose and deoxyfuconojirimycin (DFJ) on the K_M and V_{max} of the enzyme were
25 assayed in the same way in the presence of 0.25 mM fucose and 0.1 μ M DFJ, respectively.²⁸
26 Readings were performed using an Infinite 200 plate reader (TECAN) and catalytic constants

1 were calculated with the Sigma Plot 12, Systat Software.

2

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

14 In order to obtain reliable activity values, the K-FUCOSE kit (Megazyme) was
15 adapted to suit requirements. In a total volume of 250 μ l of 50 mM glycine buffer at pH 9.0,
16 the substrates 2-fucosyllactose, H-trisaccharide and Fuc- α (1,6)-GlcNAc, were incubated
17 separately at a concentration of 0.5 mM with 1.83 μ l of fucose dehydrogenase (FDH) and
18 9.15 μ l of NADP⁺ (both as supplied) in a cuvette at 37°C for 10 min. When the reaction had
19 reached a constant absorbance at 340 nm (Abs_{340}), rTfFuc1 was added to the mixture at a
20 concentration of 0.34 μ M and the formation of NADPH was followed by continuous
21 measurement of the increase in Abs_{340} . The activity of the enzyme on each substrate was
22 calculated according to the supplier's specifications from $\Delta Abs_{340}/min$ where the formation of
23 NADPH was linear over time. The experiment was repeated with different enzyme dilutions
24 (1:10; 1:100) to prove the reliability of the method.

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

1 (Carbosynth), GnGnF⁶-peptide, GalFGalF, and an *N*-glycan derived from *Crassostrea*
2 *virginica* (Eastern oyster) haemocyte treated with chicken liver α -*N*-acetylgalactosaminidase
3 and bovine β -galactosidase (both from Sigma) to reveal the underlying H epitope presenting a
4 terminal α (1,2) fucose on an unsubstituted β (1,3)-galactose (*i.e.*, histo blood group antigen H),
5 referred to here as GalF⁴² (**Fig. 3**). Activity on the A antigen substrate was assayed using the
6 K-FUCOSE kit as described above. For all other substrates, incubations were performed
7 overnight at 37°C and analysed by MALDI-TOF MS using an Autoflex Speed instrument
8 (Bruker) in positive ion mode with 6-aza-2-thiothymine (ATT) as matrix. Spectra were
9 processed with the manufacturer's software (Bruker Flexanalysis 3.3.80) using the SNAP
10 algorithm with a signal/noise threshold of 6 for MS (unsmoothed) and then manually
11 interpreted.

12

13 **rTfFuc1 activity on mucin from bovine submaxillary glands.** rTfFuc1 was incubated with
14 bovine submaxillary mucin (Sigma) in combination with the His-tagged recombinant NanH
15 sialidase (rNanH) from *T. forsythia*.¹⁵ Incubations were performed at 37°C in 50 mM glycine
16 buffer, pH 9.0, at a final concentration of 0.1 μ M of either enzyme, 0.2 mg/ml of mucin and
17 NADP⁺ and FDH as described above, in a total volume of 250 μ l. rTfFuc1 (and rNanH) was
18 added to the mixture after an initial 5-min incubation period. The release of fucose (Δ Abs₃₄₀)
19 was followed over 10 min and calculated according to the supplier's specifications from
20 Δ Abs₃₄₀/min where the formation of NADPH was linear over time.

21

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

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

10

11 **Microtiter assays of Hoechst-stained biofilms.** *T. forsythia* WT and $\Delta Tffuc1$ strains were
12 compared in respect to the biofilm formation⁴⁴ on mucin-coated polystyrene microtiter plates
13 in dependency of the strength of the BHI liquid medium. Biofilm was stained with Hoechst
14 33258 Fluorescent Stain (Thermo Scientific). Details are described in the Supplementary
15 Information.

16

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

21

22 **Disclosure and Potential Conflict of Interest**

23

24 No potential conflicts of interests were disclosed.

25

Acknowledgements

The authors thank Dr. Bernard Henrissat (CAZy) for discussing classification of the Tffucl 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).

References

1. Vos T, Flaxman AD, Naghavi M, Lozano R, Michaud C, Ezzati M, et al. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012; 380:2163-96.
2. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998; 25:134-44.
3. Haffajee AD, Socransky SS, Patel MR, Song X. Microbial complexes in supragingival plaque. *Oral Microbiol Immunol* 2008; 23:196-205.
4. Cullinan MP, Ford PJ, Seymour GJ. Periodontal disease and systemic health: current status. *Aust Dent J* 2009; 54:S62-9.
5. Posch G, Sekot G, Friedrich V, Megson ZA, Koerdt A, Messner P, et al. Glycobiology aspects of the periodontal pathogen *Tannerella forsythia*. *Biomolecules* 2012; 2:467-82.
6. Sekot G, Posch G, Oh YJ, Zayni S, Mayer HF, Pum D, et al. Analysis of the cell surface layer ultrastructure of the oral pathogen *Tannerella forsythia*. *Arch Microbiol* 2012; 194:525-39.

- 1 7. Lee SW, Sabet M, Um HS, Yang J, Kim HC, Zhu W. Identification and characterization
2 of the genes encoding a unique surface (S-) layer of *Tannerella forsythia*. *Gene* 2006;
3 371:102-11.
- 4 8. Posch G, Pabst M, Brecker L, Altmann F, Messner P, Schäffer C. Characterization and
5 scope of S-layer protein O-glycosylation in *Tannerella forsythia*. *J Biol Chem* 2011;
6 286:38714-24.
- 7 9. Sabet M, Lee SW, Nauman RK, Sims T, Um HS. The surface (S-) layer is a virulence
8 factor of *Bacteroides forsythus*. *Microbiology* 2003; 149:3617-27.
- 9 10. Sakakibara J, Nagano K, Murakami Y, Higuchi N, Nakamura H, Shimozato K, et al.
10 Loss of adherence ability to human gingival epithelial cells in S-layer protein-deficient
11 mutants of *Tannerella forsythensis*. *Microbiology* 2007; 153:866-76.
- 12 11. Honma K, Inagaki S, Okuda K, Kuramitsu HK, Sharma A. Role of a *Tannerella*
13 *for sythia* exopolysaccharide synthesis operon in biofilm development. *Microb Pathog*
14 2007; 42:156-66.
- 15 12. Douglas CO, Naylor K, Phansopa C, Frey AM, Farmilo T, Stafford GP. Physiological
16 adaptations of key oral bacteria. *Advances in Microbial Physiology* 2014; in press.
- 17 13. Stafford G, Roy S, Honma K, Sharma A. Sialic acid, periodontal pathogens and
18 *Tannerella forsythia*: stick around and enjoy the feast! *Mol Oral Microbiol* 2012; 27:11-
19 22.
- 20 14. Honma K, Mishima E, Sharma A. Role of *Tannerella forsythia* NanH sialidase in
21 epithelial cell attachment. *Infect Immun* 2011; 79:393-401.
- 22 15. Roy S, Honma K, Douglas CW, Sharma A, Stafford GP. Role of sialidase in
23 glycoprotein utilization by *Tannerella forsythia*. *Microbiology* 2011; 157:31953-202.
- 24 16. Duran-Pinedo AE, Chen T, Teles R, Starr JR, Wang X, Krishnan K, et al. Community-
25 wide transcriptome of the oral microbiome in subjects with and without periodontitis.
26 *ISME J* 2014; 8:1659-72.

- 1 17. Lühn K, Wild MK. Human deficiencies of fucosylation and sialylation affecting selectin
2 ligands. *Semin Immunopathol* 2012; 34:383-99.
- 3 18. Bode L, Jantscher-Krenn E. Structure-function relationships of human milk
4 oligosaccharides. *Adv Nutr* 2012; 3:383S-91S.
- 5 19. Katayama T, Sakuma A, Kimura T, Makimura Y, Hiratake J, Sakata K, et al. Molecular
6 cloning and characterization of *Bifidobacterium bifidum* 1,2-a-L-fucosidase (AfcA), a
7 novel inverting glycosidase (glycoside hydrolase family 95). *J Bacteriol* 2004;
8 186:4885-93.
- 9 20. Boren T, Falk P, Roth KA, Larson G, Normark S. Attachment of *Helicobacter pylori* to
10 human gastric epithelium mediated by blood group antigens. *Science* 1993; 262:1892-5.
- 11 21. Ruiz-Palacios GM, Cervantes LE, Ramos P, Chavez-Munguia B, Newburg DS.
12 *Campylobacter jejuni* binds intestinal H(O) antigen (Fuca1, 2Gal b1, 4GlcNAc), and
13 fucosyloligosaccharides of human milk inhibit its binding and infection. *J Biol Chem*
14 2003; 278:14112-20.
- 15 22. Stahl M, Friis LM, Nothaft H, Liu X, Li J, Szymanski CM, et al. L-fucose utilization
16 provides *Campylobacter jejuni* with a competitive advantage. *Proc Natl Acad Sci U S A*
17 2011; 108:7194-9.
- 18 23. Coyne MJ, Reinap B, Lee MM, Comstock LE. Human symbionts use a host-like
19 pathway for surface fucosylation. *Science* 2005; 307:1778-81.
- 20 24. Veith PD, O'Brien-Simpson NM, Tan Y, Djatmiko DC, Dashper SG, Reynolds EC.
21 Outer membrane proteome and antigens of *Tannerella forsythia*. *J Proteome Res* 2009;
22 8:4279-92.
- 23 25. Guillotin L, Lafite P, Daniellou R. Unraveling the substrate recognition mechanism and
24 specificity of the unusual glycosyl hydrolase family 29 BT2192 from *Bacteroides*
25 *thetaiotaomicron*. *Biochemistry* 2014; 53:1447-55.

- 1 26. Berteau O, McCort I, Goasdoué N, Tissot B, Daniel R. Characterization of a new alpha-
2 L-fucosidase isolated from the marine mollusk *Pecten maximus* that catalyzes the
3 hydrolysis of alpha-L-fucose from algal fucoidan (*Ascophyllum nodosum*).
4 *Glycobiology* 2002; 12:273-82.
- 5 27. Rodríguez-Díaz J, Monedero V, Yebra MJ. Utilization of natural fucosylated
6 oligosaccharides by three novel alpha-L-fucosidases from a probiotic *Lactobacillus*
7 *casei* strain. *Appl Environ Microbiol* 2011; 77:703-5.
- 8 28. Winchester B, Barker C, Baines S, Jacob GS, Namgoong SK, Fleet G. Inhibition of alpha-L-
9 fucosidase by derivatives of deoxyfuconojirimycin and deoxymannojirimycin. *Biochem*
10 *J* 1990; 265:277-82.
- 11 29. Hajishengallis G, Lamont RJ. Beyond the red complex and into more complexity: the
12 polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Mol*
13 *Oral Microbiol* 2012; 27:409-19.
- 14 30. Marsh PD. Are dental diseases examples of ecological catastrophes? *Microbiology*
15 2003; 149:279-94.
- 16 31. Marsh PD, Møter A, Devine DA. Dental plaque biofilms: communities, conflict and
17 control. *Periodontol* 2000 2011; 55:16-35.
- 18 32. Tomek MB, Neumann L, Nimeth I, Koerdt A, Andesner P, Messner P, et al. The S-layer
19 proteins of *Tannerella forsythia* are secreted via a type IX secretion system that is
20 decoupled from protein O-glycosylation. *Mol Oral Microbiol* 2014; 29:307-20.
- 21 33. Inagaki S, Onishi S, Kuramitsu HK, Sharma A. *Porphyromonas gingivalis* vesicles
22 enhance attachment, and the leucine-rich repeat BspA protein is required for invasion of
23 epithelial cells by *Tannerella forsythia*. *Infect Immun* 2006; 74:5023-8.
- 24 34. Larsbrink J, Rogers TE, Hemsworth GR, McKee LS, Tausin AS, Spadiut O, et al. A
25 discrete genetic locus confers xyloglucan metabolism in select human gut
26 *Bacteroidetes*. *Nature* 2014; 506:498-502.

- 1 35. Martens EC, Koropatkin NM, Smith TJ, Gordon JI. Complex glycan catabolism by the
2 human gut microbiota: the *Bacteroidetes* Sus-like paradigm. *J Biol Chem* 2009;
3 284:24673-7.
- 4 36. Thompson H, Homer KA, Rao S, Booth V, Hosie AH. An orthologue of *Bacteroides*
5 *fragilis* NanH is the principal sialidase in *Tannerella forsythia*. *J Bacteriol* 2009;
6 191:3623-8.
- 7 37. Cheng HR, Jiang N. Extremely rapid extraction of DNA from bacteria and yeasts.
8 *Biotechnol Lett* 2006; 28:55-9.
- 9 38. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. Cold Spring Harbor Laboratory
10 Press, Cold Spring Harbor, New York 1989.
- 11 39. Laemmli UK. Cleavage of structural proteins during the assembly of the head of
12 bacteriophage T4. *Nature* 1970; 227:680-5.
- 13 40. Leonard R, Rendic D, Rabouille C, Wilson IB, Preat T, Altmann F. The *Drosophila*
14 fused lobes gene encodes an *N*-acetylglucosaminidase involved in *N*-glycan processing.
15 *J Biol Chem* 2006; 281:4867-75.
- 16 41. McIlvaine TA. A buffer solution for colorimetric comparison. *J Biol Chem* 1921;
17 49:183-6.
- 18 42. Kurz S, Jin C, Hykollari A, Gregorich D, Giomarelli B, Vasta GR, et al. Hemocytes and
19 plasma of the eastern oyster (*Crassostrea virginica*) display a diverse repertoire of
20 sulfated and blood group A-modified *N*-glycans. *J Biol Chem* 2013; 288:24410-28.
- 21 43. Bradford MM. A rapid and sensitive method for the quantitation of microgram
22 quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;
23 72:248-54.
- 24 44. Honma K, Mishima E, Inagaki S, Sharma A. The OxyR homologue in *Tannerella*
25 *forseythia* regulates expression of oxidative stress responses and biofilm formation.
26 *Microbiology* 2009; 155:1912-22.

1 45. Stafford P, Higham J, Pinnock A, Murdoch C, Douglas CW, Stafford GP, et al.
2 Gingipain-dependent degradation of mammalian target of rapamycin pathway proteins
3 by the periodontal pathogen *Porphyromonas gingivalis* during invasion. Mol Oral
4 Microbiol 2013; 28:366-78.

5

6

7

1 **Figure legends**

2

3 **Figure 1.** SDS PAGE (A.) and Western immunoblot (B.) of total cell extracts from *T.*
4 *forsythia* WT (lane 2) and $\Delta Tffuc1$ strains (lane 3) and of the His₆-tagged rTfFuc1 as purified
5 from *E. coli* (lane 4), used for activity studies and to raise a polyclonal anti-TfFuc1 antiserum.
6 Western immunoblotting using the anti-TfFuc1 antiserum recognized the protein (~51 kDa)
7 specifically in the WT strain (lane 2) and indicated absence of the protein in the $\Delta Tffuc1$ strain
8 (lane 3), proving that the enzyme was effectively knocked-out. In the preparation of rTfFuc 1
9 (B., lane 4), the polyclonal antiserum recognizes also minor contaminating *E. coli* proteins not
10 visible on the SDS-PAGE gel (A., lane 4). Mm; PageRuler Plus prestained protein ladder
11 (Thermo Scientific).

12

13 **Figure 2.** pH profile of rTfFuc1 using 4-nitrophenyl- α -L-fucopyranoside (pNP-fucose) as a
14 substrate. Activity was measured as the increase in Abs₄₀₅ due to the released 4-nitrophenol
15 product. Citrate/phosphate buffer (0.1 M) was used to assay the pH range from 3-8, 50 mM
16 glycine buffer was used for the pH range from 8-10.25.

17

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

21

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

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

6

7 **Figure 6.** rTfFuc1 was incubated with mucin from bovine submaxillary glands and the release
8 of fucose was measured with the K-FUCOSE kit. When incubations were performed in
9 conjunction with the rNanH sialidase, a slow steady increase in the Abs₃₄₀ was observed. The
10 activity was calculated over a period of 300 s where the data points fitted a linear regression
11 with an r^2 of 0.98. No activity could be detected, when rTfFuc1 was incubated alone with the
12 mucin. The Δ Abs₃₄₀ lead to an irregular data set with a very low r^2 value of 0.4.

13

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

23

1 **Table 1.** rFuc1 activity on 4-nitrophenyl- α -L-fucopyranoside (pNP-fucose). Catalytic
 2 constants K_M and V_{max} and the inhibitory effect of DFJ and L-fucose were measured using the
 3 colorimetric substrate pNP-fucose within a concentration range from 0.01 to 50 mM at 22°C
 4 in glycine buffer at pH 9.0

5

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

6

7 *4-nitrophenyl- α -L-fucopyranoside

8 ** μ mol/min/mg of enzyme

9 ***deoxyfuconojirimycin

10

11

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	-

* μ mol/min/mg of enzyme

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant characteristic(s)	Source
<i>Escherichia coli</i> DH5 α	F ⁻ ϕ 80d <i>lacZ</i> M15 (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1</i> <i>hsdR17</i> (rK ⁻ mK ⁻) <i>phoA supE44 thi-1 gyrA96 relA1⁻</i>	Invitrogen
<i>Escherichia coli</i> BL21 (DE)	F ⁻ , <i>ompT</i> , <i>hsdS</i> (<i>rB⁻mB⁻</i>), <i>gal</i> , <i>dcm</i> (DE3)	Invitrogen
<i>Tannerella forsythia</i> ATCC 43037	Wild-type isolate	American Type Culture Collection, USA
<i>T. forsythia</i> Δ <i>Tffuc1</i>	<i>T. forsythia</i> knockout of the <i>Tffuc1</i> gene; Erm ^r	This study
pET-22b (+)	Expression vector with a His ₆ -tag, Amp ^r	Novagen
pJET1.2- <i>Tffuc1_</i> ko	pJET1.2 carrying the <i>Tffuc1_ermF-AM</i> knockout cassette	This study

Table 4. Oligonucleotide primers used for PCR amplification reactions^a

Primers	Sequence (5' → 3') ^a
1	gcgg <u>CATATG</u> AAAACAAGAACATTACTTCTTTGTG
2	gcta <u>CTCGAG</u> TTTTAGAGGCAATTCATTGGCAAATG
3	GACCAAGCTGCAGGCCATCATCGATGTGCTCAAC
A	<i>GAAGCTATCGGGGGTACCTCCCCGGG</i> -AGAATAATTTTTGTTTATTACTAAAAATAACG
5	<i>GCTTCGGGGATCCTCTAGCCCCGGG</i> -CAGAAATATCTTTATGAAACATCCTATTGATGGGGTG
6	GCTCAGCCAGCCGATAGTTACTTTTTTCGTTATGTGTTCCC
7 ^b	CGTTATTTTTTAGTAATAAACAAAATTATTCT-<u>CCCGGGGGAGGTACCCCGATAGCTTC</u>
8 ^b	CACCCCATCAATAGGATGTTTCATAAAGATATTTCTG-<u>CCCGGGGGCTAGAGGATCCCGAAGC</u>
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

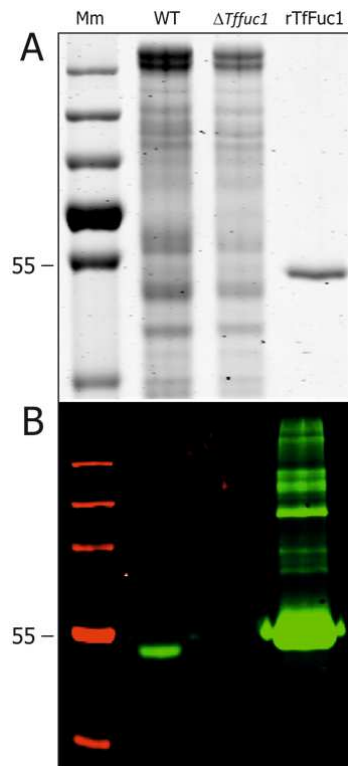


Figure 1. of Megson *et al.*

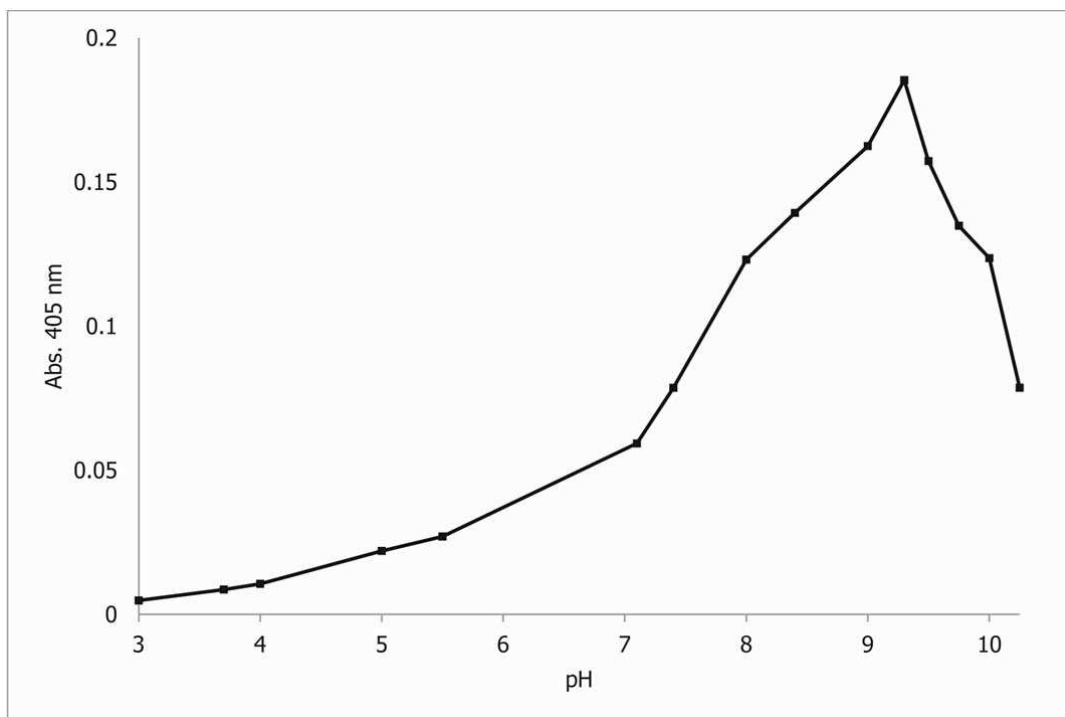


Figure 2. of Megson *et al.*

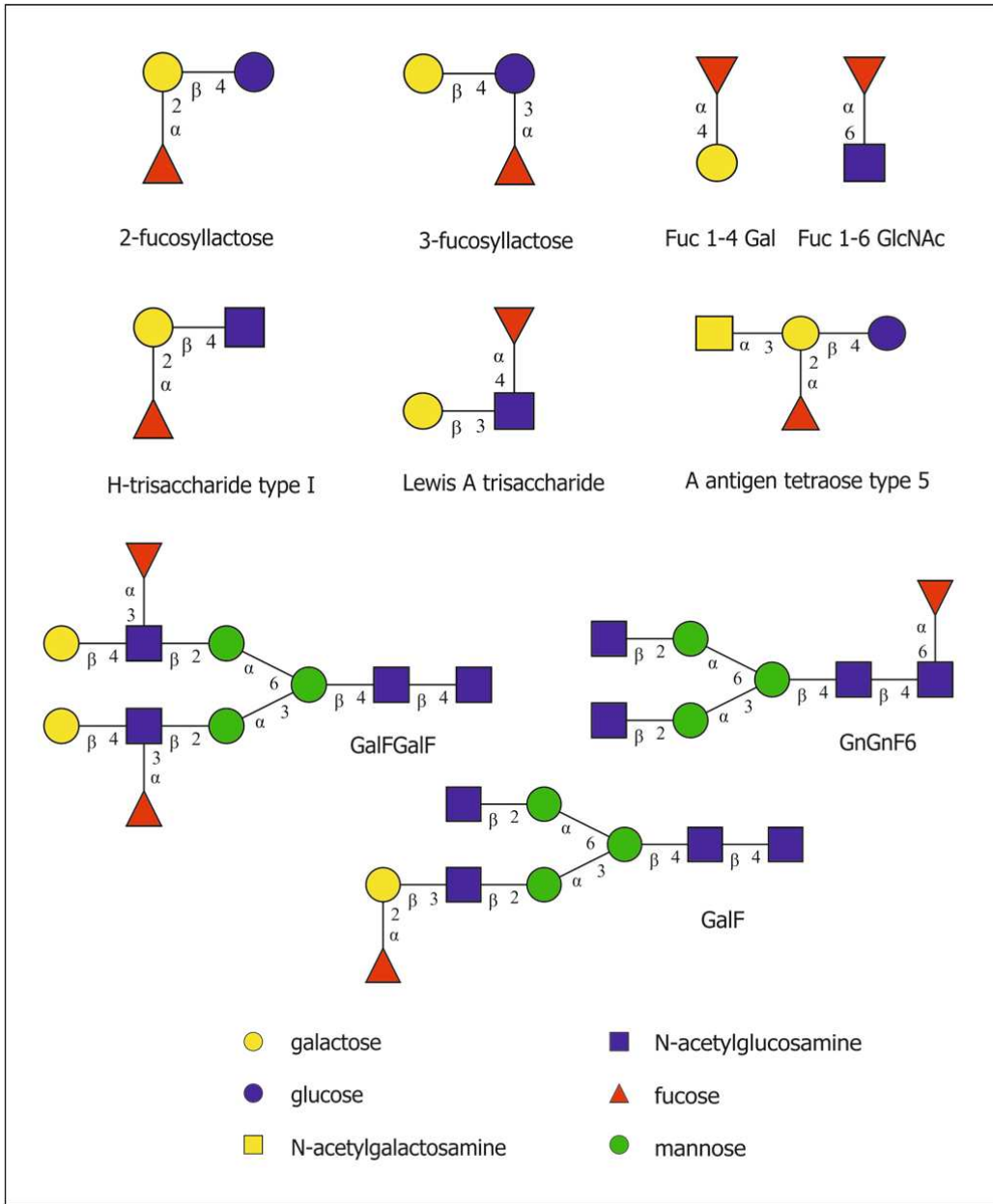


Figure 3. of Megson *et al.*

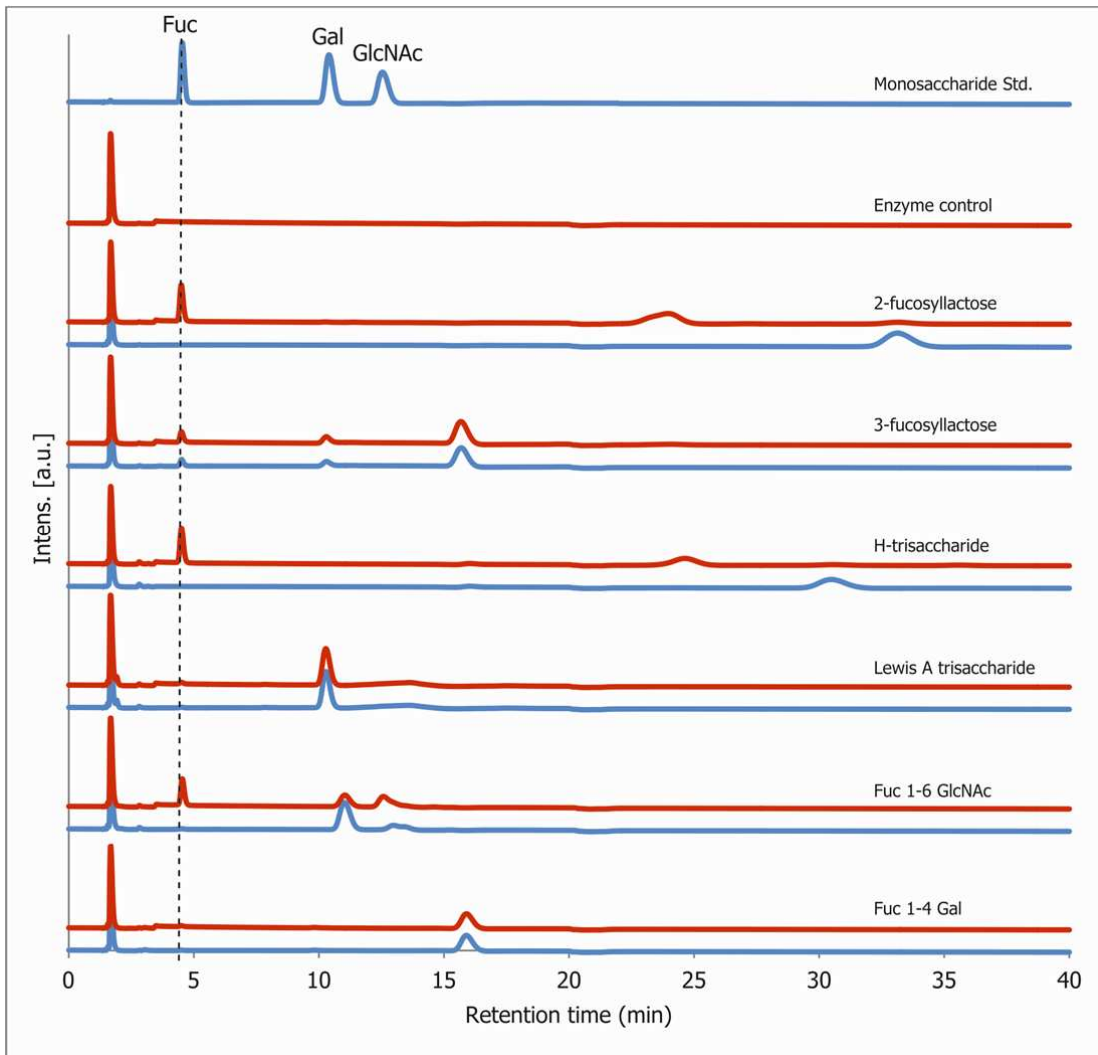


Figure 4. of Megson *et al.*

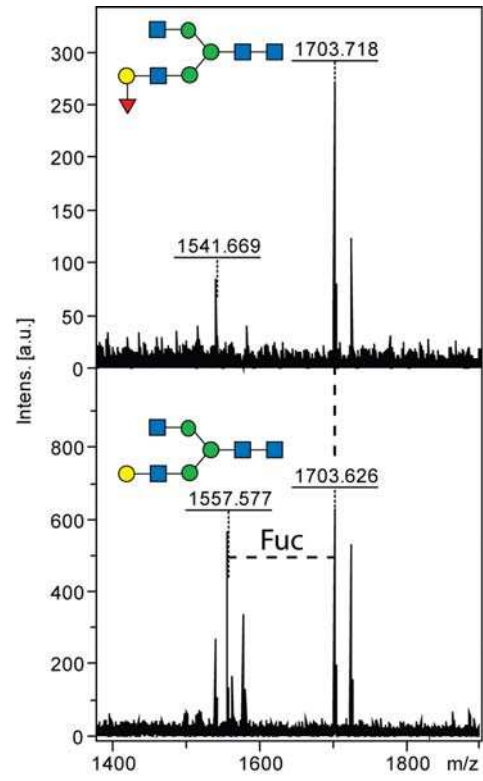


Figure 5. of Megson *et al.*

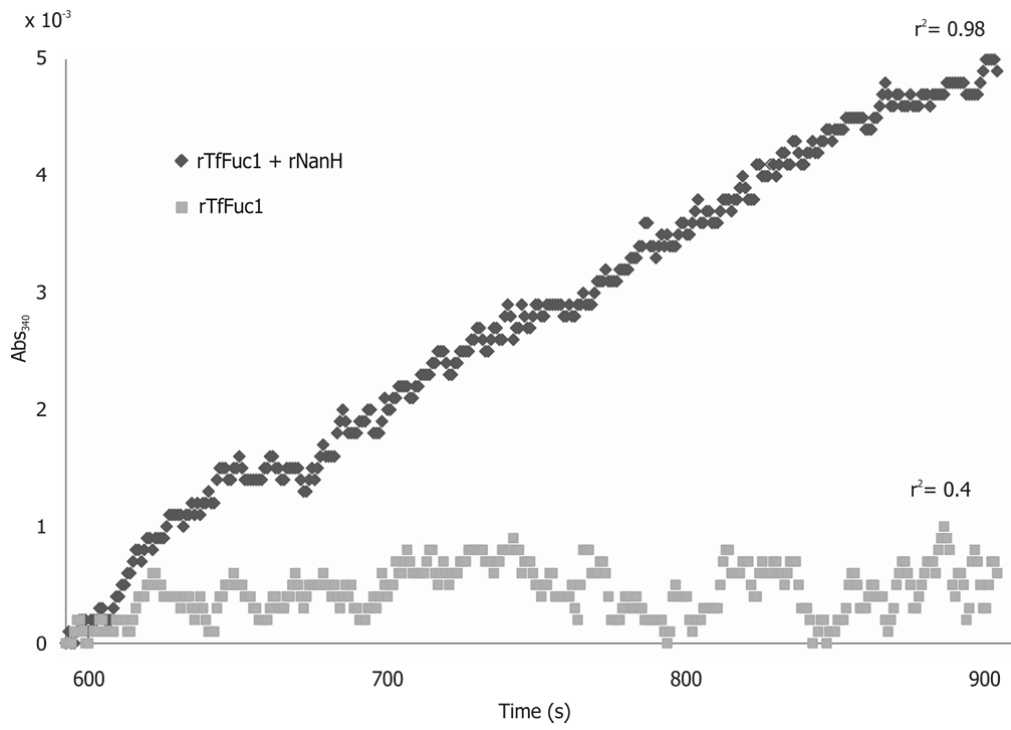


Figure 6. of Megson *et al.*

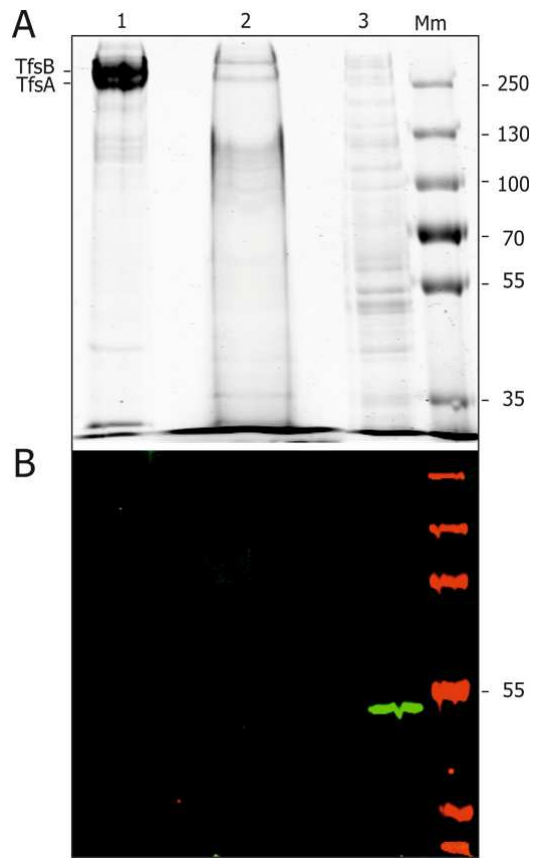


Figure 7. of Megson *et al.*

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

Submitted to Virulence

Research Paper

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

Z. A. Megson¹, A. Koerdt¹, H. Schuster¹, R. Ludwig², B. Janesch¹, A. Frey³,
K. Naylor³, I. B.H. Wilson⁴, G. P. Stafford³, P. Messner¹ and C. Schäffer^{1*}

¹Department of NanoBiotechnology, *NanoGlycobiology* unit, Universität für Bodenkultur
Wien, Vienna, Austria

²Department of Food Science and Technology, Universität für Bodenkultur Wien, Vienna,
Austria

³Integrated BioSciences, School of Clinical Dentistry, University of Sheffield, Sheffield, UK

⁴Department of Chemistry, Universität für Bodenkultur Wien, Vienna, Austria

*Correspondence to: Christina Schäffer; E-mail: christina.schaeffer@boku.ac.at

1 **Abstract**

2

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

22

1 Introduction

2

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

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

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

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

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

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

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

3 Here, we describe the molecular cloning and characterization of BFO_2737, which we
4 named Tffuc1. This protein has previously been reported to form part of an outer membrane
5 preparation of *T. forsythia*²⁴ and, thus, was a good candidate to be involved in host-pathogen
6 interactions. Tffuc1 is a 446-amino acid protein with a theoretical pI and molecular mass of
7 6.9 and 50.8 kDa, respectively. It is the first fucosidase in this organism to be characterized to
8 date. The enzyme was shown here to be an α (1,2)-fucosidase and also possesses an α (1,6)
9 specificity on small unbranched substrates. It is a predicted periplasmic protein, possibly
10 playing a role in the breakdown of small oligosaccharides. It is, to the best of our knowledge,
11 the first glycosyl hydrolase in its family (GH29) reported to be a specific α (1,2)-fucosidase.

12

13 **Results**

14

15 **Enzymatic characterization of rTffuc1.** The *Tffuc1* gene was cloned into pET22-b(+)
16 vector and expressed in *E. coli* as a C-terminally His₆-tagged protein, which enabled
17 purification via nickel affinity chromatography (**Fig. 1**). The enzymatic activity was then
18 tested using the standard colorimetric α -fucosidase substrate 4-nitrophenyl- α -L-
19 fucopyranoside (pNP-fucose) at 22°C in a range of different pH values and in the presence of
20 MgCl₂, KCl and NaCl, in order to establish its pH optimum and cation dependence,
21 respectively. By stopping the reaction with the addition of an alkaline buffer at pH 11.4, it
22 was ensured that all wells were at the same pH for consequent absorbance readings. The
23 activity of the enzyme was seen to start to plateau at the neutral to alkaline pH range and was
24 considered most active at pH 9.0, assayed in glycine buffer, and not at pH 9.25 where the
25 activity suddenly peaks and then rapidly decreases thereafter. The activity remained largely
26 unaffected by the presence of cations at the two concentrations tested (results not shown). The

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

6
7 **Substrate linkage specificity of rTffuc1.** To determine the enzyme linkage specificity,
8 rTffuc1 was incubated with a set of different fucosylated substrates of defined structure
9 representing a range of fucose linkages available on host glycoproteins and on oral surfaces
10 (**Fig. 3**). The reaction products obtained after overnight incubation were analysed using high-
11 pH anion-exchange chromatography with pulsed amperometric detection (HPAEC) where the
12 release of fucose was confirmed by comparison with the retention time of the standard
13 monosaccharide and of a substrate standard after overnight incubation at 37°C. The enzyme
14 was seen to be active on both $\alpha(1,2)$ fucose containing substrates, 2-fucosyllactose and H-
15 trisaccharide, and on the $\alpha(1,6)$ fucose disaccharide α -L-Fuc-(1,6)- β -D-GlcNAc, although this
16 latter reaction did not reach completion, indicating weak specificity for this linkage. The
17 $\alpha(1,3)$ and $\alpha(1,4)$ linkages were not cleaved on 3-fucosyllactose and the Lewis A
18 trisaccharide, respectively. The enzyme was also inactive on the substrate α -L-Fuc-(1,4)- β -D-
19 Gal, added as a second disaccharide control to prove that the $\alpha(1,6)$ activity was not due to
20 differences in substrate length (**Fig. 4**).

21 In order to obtain accurate activity values on the cleaved substrates, the K-FUCOSE
22 kit from Megazyme was used, coupled to the enzymatic reaction with rTffuc1. First, FDH,
23 which also has an alkaline pH optimum, and $NADP^+$ were added to the substrate solution
24 reaction mixture in order to convert any free fucose already present in the sample to L-fucono-
25 1,5-lactone by the reduction of $NADP^+$ to NADPH ($\epsilon_{340} = 6.022 \text{ mM}^{-1} \text{ cm}^{-1}$). rTffuc1 was then
26 added to the mixture and the reaction was monitored by following the increase in Abs_{340} . The

1 activity was calculated from where the formation of NADPH was linear over time. The
2 enzyme was most active on 2-fucosyllactose and H-trisaccharide with specific activities of
3 0.8 U/mg and 0.6 U/mg, respectively. The activity on the $\alpha(1,6)$ disaccharide was
4 significantly lower at 0.35 U/mg (**Table 2**).

5 The activity of the enzyme on the various substrates could be calculated
6 approximately (as some loss of material occurred during sample preparation) from the HPLC
7 experiments after 1-h incubation periods (results not shown) and was found to be markedly
8 lower than that observed with the K-FUCOSE kit, indicating that free fucose, which is
9 consumed in the latter, could be inhibiting the enzymatic activity significantly. In order to
10 determine the extent of such an effect, measurement of K_M and V_{max} values were repeated
11 with pNP-fucose in the presence of either 0.25 mM L-fucose or 0.1 μ M deoxyfuconojirimycin
12 (DFJ), which is a strong fucosidase inhibitor.²⁸ The enzyme was competitively inhibited by
13 both fucose and DFJ as the V_{max} remained largely unaffected but the K_M value increased from
14 0.67 mM to 16.5 mM and 28.3 mM, respectively (**Table 1**).

15 Further, the ability of the enzyme to cleave fucose residues off more complex
16 natural glycans and those on branched sugar residues was assayed (compare with **Fig. 3**). As
17 expected, the enzyme was unable to cleave the $\alpha(1,3)$ fucose linkage on GalFGalF-pep,
18 included in the assays as a trace amount of activity could be observed when using 3-
19 fucosyllactose, as measured by the K-FUCOSE kit. The enzyme was also not able to cleave
20 the core α -1,6 fucose linkage on GnGnF⁶-pep nor the branched $\alpha(1,2)$ -linked fucose on the
21 A antigen. The non-branched $\alpha(1,2)$ fucose linkage present on the Eastern oyster substrate,
22 however, was cleaved off the substrate GalF, seen by the loss of a fucose residue in the MS
23 spectra of the substrate. The major m/z 1703 glycan ($[M+H]^+$) was approximately 50%
24 digested to a defucosylated species of m/z 1557 after overnight incubation with the enzyme
25 (**Fig. 5**). The enzyme is, therefore, able to cleave off fucose residues which are $\alpha(1,2)$ linked
26 on more complex glycans only when in a terminal unbranched position and is unable to cleave

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

2

3 **rTfFuc1 activity on bovine submaxillary mucin.** rTfFuc1 was incubated with mucin from
4 bovine submaxillary glands and the release of fucose was measured with the K-FUCOSE kit.
5 Incubation was performed also in combination with rNanH from *T. forsythia* and activities
6 were calculated from the slope of ΔAbs_{340} where it was linear over time. During the assayed
7 incubation period of 10 min, no activity could be detected when rTfFuc1 was incubated alone
8 with mucin. The ΔAbs_{340} lead to an irregular data set with a very low r^2 value. However,
9 fucose release was detectable when the incubation was performed in conjunction with the
10 rNanH sialidase, presenting a slow but steady increase in the Abs_{340} . The activity was
11 calculated over a period of 300 s where the data points fitted a linear regression with an r^2 of
12 0.98 (**Fig. 6**). The experiment was repeated several times and yielded an activity of 24
13 ± 4 mU/mg. rTfFuc1 might cleave fucose off mucin over longer periods of time, but the data
14 shows a significantly higher activity when sialic acid residues are first removed from mucin,
15 indicating that the fucosidase TfFuc1 could work downstream from the sialidase in *T.*
16 *forsythia* and presumably cooperate with other glycosidases in the degradation of complex
17 glycans.

18

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

1 comprising both the cytoplasmic and periplasmic content (**Fig. 7**), arguing against surface
2 localization of the Tffuc1 enzyme.

3

4 **Discussion**

5

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

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

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

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

9 During the course of this study, it became clear that one of the issues possibly
10 underlining our observations was the enzyme's cellular localization. Even though TfFuc1 was
11 found previously to be present in the outer membrane proteome of the pathogen,²⁴ localization
12 of the enzyme on the surface of *T. forsythia* cells by fluorescent immunolabelling was not
13 successful (data not shown; see Experimental Procedures in the Supplementary Information)
14 and cell fractionation also showed the detectable protein to be found in the non-membrane
15 associated fraction (**Fig. 7**). This would be in agreement with bioinformatics interrogation
16 indicating that, while it has a Sec-dependent secretion signal (as predicted by SignalP 4.1
17 Server), it is not predicted to reside in the outer membrane or be secreted, nor does it contain a
18 T9SS C-terminal secretion signal.³² The difference in location of TfFuc1 between the present
19 study and the outer membrane proteomics experiment²⁴ can be reconciled when considering
20 that in the latter study, cross-contamination of individual proteins between cellular fractions
21 was not investigated.

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

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

5 This notion that the *T. forsythia* fucosidase TfFuc1 plays an internal role was
6 corroborated when we tested the effect of the $\Delta Tffuc1$ mutation on the ability of *T. forsythia* to
7 interact with and invade human oral epithelial cells using an antibiotic protection assay on the
8 oral epithelial cell line H357.³³ We found no significant differences in the ability of the
9 $\Delta Tffuc1$ to invade these human cells as compared to the WT strain (**Fig. S1**), indicating that
10 TfFuc1 has no effect on epithelial cell-invasion under the conditions tested. In addition, the
11 mutant did not show hindered biofilm formation when cultured on bovine submaxillary
12 mucin, contrary to the *T. forsythia NanH* sialidase mutant,¹⁵ but showed a slightly increased
13 biofilm formation (**Fig. S2**).

14 Our data supports the idea of a periplasmic fucosidase involved in the final
15 breakdown of small substrates that have been internalized, possibly owing to the action of
16 exoglycosidases and endoglycosidases which break-down larger glycans on the outside of the
17 cell.^{34, 35} TfFuc1 would possibly then be able to exert its full potential freeing both $\alpha(1,2)$ and
18 $\alpha(1,6)$ fucoses on small linear substrates. These findings are also in agreement with the
19 hypothesis that the fucosidase acts downstream of the sialidases, which have been shown to
20 act on whole glycoproteins on bacterial and host surfaces.^{14, 15, 36} The sialic acid would,
21 therefore, already be missing as smaller parts of the glycan are transported inside the cell.

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

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

6
7 Overall, our data suggest that Tffuc1 is a unique α -L-(1,2)-fucosidase which could potentially
8 contribute to fucose utilization in *T. forsythia*. In order to better elucidate this role and rule out
9 any redundancy in the system, two further annotated fucosidases in *T. forsythia*, BFO_1182
10 and BFO_3101, together with the annotated fucose permease, BFO_0307, are now being
11 investigated to elucidate the role of fucose in the physiology of *T. forsythia*.

12

13 **Experimental Procedures**

14

15 **Bacterial strains, medium and culture conditions.** *T. forsythia* wild-type (WT) strain
16 ATCC 43037 (American Type Culture Collection) and the knock-out mutant $\Delta Tffuc1$ were
17 grown anaerobically at 37°C for 4-7 d in brain heart infusion (BHI) broth or 0.8% (w/v) BHI
18 agar, supplemented with *N*-acetylmuramic acid (NAM), horse serum and gentamycin as
19 described previously.³² *Escherichia coli* DH5 α and BL21 (DE3) (Invitrogen) were cultivated
20 in selective Luria Bertani (LB) medium (agar and broth) supplemented with 100 μ g/ml
21 ampicillin (Amp). All strains and plasmids used in the course of this study are summarised in
22 **Table 3.**

23

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

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

12

13 **Recombinant production of His₆-tagged Tffuc1.** The *Tffuc1* gene was amplified from the
14 chromosome of *T. forsythia* ATCC 43037 with a fused C-terminal His₆-tag by PCR using
15 primer pair 1/2 (**Table 4**). The His₆-tagged amplification product was digested using
16 restriction enzymes NdeI/XhoI and cloned into NdeI/XhoI-linearized pET22-b (Novagen).
17 The corresponding plasmid was transformed into *E. coli* BL21 (DE3) cells for protein
18 expression. Freshly transformed cells were grown in two 400-ml Erlenmeyer flasks to an
19 OD₆₀₀ of 0.4-0.5 in the presence of 100 µg/ml of Amp at which point protein expression was
20 induced with a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and
21 cultures were shaken (200 rpm) overnight at 18°C. Cells were harvested by centrifugation
22 (6,500 g, 20 min, 4°C).

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

1 and the His₆-tagged protein was purified using an imidazole gradient in buffer A; 25 mM
2 imidazole (10 ml), 50 mM imidazole (10 ml), followed by five elution steps with 500 µl of
3 250 mM imidazole in buffer A. Eluted fractions containing the purified recombinant protein,
4 rTfFuc1, as determined by SDS-PAGE analysis, were pooled and dialysed overnight at 4°C
5 against 3 l of 10 mM phosphate buffer, pH 8.0. The volume was then reduced 5-fold using a
6 concentration centrifuge yielding a protein concentration of 0.35 mg/ml (as determined by
7 Nanodrop) in 50 mM phosphate buffer.

8

9 **Construction of a *T. forsythia* $\Delta Tffuc1$ knock-out strain.** Disruption of the *Tffuc1* gene in
10 *T. forsythia* was performed by gene knockout, as described previously.³² The *Tffuc1* gene is
11 not part of an operon, thus, downstream effects due to the chosen mutation strategy are not
12 expected to occur. Briefly, the flanking genomic regions (1,000 bp) up-stream and down-
13 stream of *Tffuc1* were amplified using primer pairs 3/4 and 5/6, respectively (**Table 4**). The
14 two resulting fragments were joined with the erythromycin resistance gene *ermF-ermAM*
15 (amplified using primer pair 7/8) by overlap extension PCR and sub-cloned into the blunt-end
16 cloning vector pJET1.2 (Thermo Scientific), resulting in pJET1.2/*Tffuc1*_ko. Approximately
17 5 µg of the knockout cassette was transferred by electroporation into 100 µl of competent *T.*
18 *forseythia* cells. Cells were regenerated in BHI medium for 24 h before plating on BHI agar
19 plates containing erythromycin (10 µg/ml) as a selection marker. Single colonies were picked
20 and used for inoculation of liquid BHI medium. Genomic DNA of the new $\Delta Tffuc1$ mutants
21 were isolated as mentioned above and the absence of the *Tffuc1* gene and the correct
22 integration of the erythromycin resistance gene (upstream and downstream) was evaluated by
23 PCR using primer pairs 1/2, 9/10, and 11/12, respectively (**Table 4**). Absence of the enzyme
24 in the $\Delta Tffuc1$ strain was also confirmed by Western immunoblotting of the total cell extract
25 separated by SDS-PAGE using TfFuc1-specific polyclonal antiserum (**Fig. 1**).

1 **General and analytical methods.** SDS-PAGE was carried out according to a standard
2 protocol using a Protean II electrophoresis apparatus (Bio-Rad).³⁹ Protein bands were
3 visualized with Coomassie Brilliant Blue G 250 (CBB) staining reagent. For Western
4 immunoblotting of proteins onto a nitrocellulose membrane (PeqLab), a Mini Trans-Blot Cell
5 (Bio-Rad) was used. Detection of the His₆-tag fused to rTfFuc1 and detection of TfFuc1 was
6 done with the Li-Cor Odyssey Infrared Imaging System using an anti-His₆ mouse antibody
7 (Roche) or TfFuc1-specific polyclonal antiserum raised in mice (EF-BIO), respectively, both
8 in combination with goat anti-mouse IgGIR Dye 800CW conjugate (Li-Cor).

9
10 **Enzymatic characterization of rTfFuc1.** Enzymatic characterization of rTfFuc1 was
11 performed essentially as described elsewhere.⁴⁰ 0.17 μM of purified, recombinant enzyme
12 was incubated with 5 mM of the colorimetric substrate 4-nitrophenyl-α-L-fucopyranoside
13 (pNP-fucose) (Sigma) at a range of different pH values (3-10.25) and cation concentrations in
14 96-well plates at 22°C in a total volume of 40 μl. The enzymatic reaction was stopped after
15 3 min by addition of 260 μl of phosphate buffer, pH 11.4. Citrate/phosphate buffer (0.1 M)
16 was used to assay the pH range from 3-8,⁴¹ 50 mM glycine buffer was used for a pH range
17 from 8-10. The effect of MgCl₂ (5 mM, 10 mM), KCl (5 mM, 10 mM) and NaCl (50 mM,
18 150 mM) on the enzyme's activity was assayed in the same way in 50 mM glycine buffer,
19 pH 9.

20 A 4-nitrophenol standard curve was made by measuring the absorbance at 405 nm
21 (Abs₄₀₅) of 0, 4, 8, 12, 16, 20 and 24 nmol of 4-nitrophenol per well in 300 μl of phosphate
22 buffer, pH 11.4. The K_M and V_{max} catalytic constants were calculated at pH 9.0 in
23 50 mM glycine buffer at 22°C in the presence of 0.01 to 50 mM pNP-fucose. The inhibitory
24 effect of fucose and deoxyfuconojirimycin (DFJ) on the K_M and V_{max} of the enzyme were
25 assayed in the same way in the presence of 0.25 mM fucose and 0.1 μM DFJ, respectively.²⁸
26 Readings were performed using an Infinite 200 plate reader (TECAN) and catalytic constants

1 were calculated with the Sigma Plot 12, Systat Software.

2

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

14 In order to obtain reliable activity values, the K-FUCOSE kit (Megazyme) was
15 adapted to suit requirements. In a total volume of 250 μ l of 50 mM glycine buffer at pH 9.0,
16 the substrates 2-fucosyllactose, H-trisaccharide and Fuc- α (1,6)-GlcNAc, were incubated
17 separately at a concentration of 0.5 mM with 1.83 μ l of fucose dehydrogenase (FDH) and
18 9.15 μ l of NADP⁺ (both as supplied) in a cuvette at 37°C for 10 min. When the reaction had
19 reached a constant absorbance at 340 nm (Abs₃₄₀), rTfFuc1 was added to the mixture at a
20 concentration of 0.34 μ M and the formation of NADPH was followed by continuous
21 measurement of the increase in Abs₃₄₀. The activity of the enzyme on each substrate was
22 calculated according to the supplier's specifications from Δ Abs₃₄₀/min where the formation of
23 NADPH was linear over time. The experiment was repeated with different enzyme dilutions
24 (1:10; 1:100) to prove the reliability of the method.

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

1 (Carbosynth), GnGnF⁶-peptide, GalFGalF, and an *N*-glycan derived from *Crassostrea*
2 *virginica* (Eastern oyster) haemocyte treated with chicken liver α -*N*-acetylgalactosaminidase
3 and bovine β -galactosidase (both from Sigma) to reveal the underlying H epitope presenting a
4 terminal α (1,2) fucose on an unsubstituted β (1,3)-galactose (*i.e.*, histo blood group antigen H),
5 referred to here as GalF⁴² (**Fig. 3**). Activity on the A antigen substrate was assayed using the
6 K-FUCOSE kit as described above. For all other substrates, incubations were performed
7 overnight at 37°C and analysed by MALDI-TOF MS using an Autoflex Speed instrument
8 (Bruker) in positive ion mode with 6-aza-2-thiothymine (ATT) as matrix. Spectra were
9 processed with the manufacturer's software (Bruker Flexanalysis 3.3.80) using the SNAP
10 algorithm with a signal/noise threshold of 6 for MS (unsmoothed) and then manually
11 interpreted.

12

13 **rTfFuc1 activity on mucin from bovine submaxillary glands.** rTfFuc1 was incubated with
14 bovine submaxillary mucin (Sigma) in combination with the His-tagged recombinant NanH
15 sialidase (rNanH) from *T. forsythia*.¹⁵ Incubations were performed at 37°C in 50 mM glycine
16 buffer, pH 9.0, at a final concentration of 0.1 μ M of either enzyme, 0.2 mg/ml of mucin and
17 NADP⁺ and FDH as described above, in a total volume of 250 μ l. rTfFuc1 (and rNanH) was
18 added to the mixture after an initial 5-min incubation period. The release of fucose (Δ Abs₃₄₀)
19 was followed over 10 min and calculated according to the supplier's specifications from
20 Δ Abs₃₄₀/min where the formation of NADPH was linear over time.

21

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

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

10

11 **Microtiter assays of Hoechst-stained biofilms.** *T. forsythia* WT and $\Delta Tffuc1$ strains were
12 compared in respect to the biofilm formation⁴⁴ on mucin-coated polystyrene microtiter plates
13 in dependency of the strength of the BHI liquid medium. Biofilm was stained with Hoechst
14 33258 Fluorescent Stain (Thermo Scientific). Details are described in the Supplementary
15 Information.

16

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

21

22 **Disclosure and Potential Conflict of Interest**

23

24 No potential conflicts of interests were disclosed.

25

Acknowledgements

The authors thank Dr. Bernard Henrissat (CAZy) for discussing classification of the Tffucl 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).

References

1. Vos T, Flaxman AD, Naghavi M, Lozano R, Michaud C, Ezzati M, et al. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012; 380:2163-96.
2. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998; 25:134-44.
3. Haffajee AD, Socransky SS, Patel MR, Song X. Microbial complexes in supragingival plaque. *Oral Microbiol Immunol* 2008; 23:196-205.
4. Cullinan MP, Ford PJ, Seymour GJ. Periodontal disease and systemic health: current status. *Aust Dent J* 2009; 54:S62-9.
5. Posch G, Sekot G, Friedrich V, Megson ZA, Koerdt A, Messner P, et al. Glycobiology aspects of the periodontal pathogen *Tannerella forsythia*. *Biomolecules* 2012; 2:467-82.
6. Sekot G, Posch G, Oh YJ, Zayni S, Mayer HF, Pum D, et al. Analysis of the cell surface layer ultrastructure of the oral pathogen *Tannerella forsythia*. *Arch Microbiol* 2012; 194:525-39.

- 1 7. Lee SW, Sabet M, Um HS, Yang J, Kim HC, Zhu W. Identification and characterization
2 of the genes encoding a unique surface (S-) layer of *Tannerella forsythia*. *Gene* 2006;
3 371:102-11.
- 4 8. Posch G, Pabst M, Brecker L, Altmann F, Messner P, Schäffer C. Characterization and
5 scope of S-layer protein O-glycosylation in *Tannerella forsythia*. *J Biol Chem* 2011;
6 286:38714-24.
- 7 9. Sabet M, Lee SW, Nauman RK, Sims T, Um HS. The surface (S-) layer is a virulence
8 factor of *Bacteroides forsythus*. *Microbiology* 2003; 149:3617-27.
- 9 10. Sakakibara J, Nagano K, Murakami Y, Higuchi N, Nakamura H, Shimozato K, et al.
10 Loss of adherence ability to human gingival epithelial cells in S-layer protein-deficient
11 mutants of *Tannerella forsythensis*. *Microbiology* 2007; 153:866-76.
- 12 11. Honma K, Inagaki S, Okuda K, Kuramitsu HK, Sharma A. Role of a *Tannerella*
13 *for sythia* exopolysaccharide synthesis operon in biofilm development. *Microb Pathog*
14 2007; 42:156-66.
- 15 12. Douglas CO, Naylor K, Phansopa C, Frey AM, Farmilo T, Stafford GP. Physiological
16 adaptations of key oral bacteria. *Advances in Microbial Physiology* 2014; in press.
- 17 13. Stafford G, Roy S, Honma K, Sharma A. Sialic acid, periodontal pathogens and
18 *Tannerella forsythia*: stick around and enjoy the feast! *Mol Oral Microbiol* 2012; 27:11-
19 22.
- 20 14. Honma K, Mishima E, Sharma A. Role of *Tannerella forsythia* NanH sialidase in
21 epithelial cell attachment. *Infect Immun* 2011; 79:393-401.
- 22 15. Roy S, Honma K, Douglas CW, Sharma A, Stafford GP. Role of sialidase in
23 glycoprotein utilization by *Tannerella forsythia*. *Microbiology* 2011; 157:31953-202.
- 24 16. Duran-Pinedo AE, Chen T, Teles R, Starr JR, Wang X, Krishnan K, et al. Community-
25 wide transcriptome of the oral microbiome in subjects with and without periodontitis.
26 *ISME J* 2014; 8:1659-72.

- 1 17. Lühn K, Wild MK. Human deficiencies of fucosylation and sialylation affecting selectin
2 ligands. *Semin Immunopathol* 2012; 34:383-99.
- 3 18. Bode L, Jantscher-Krenn E. Structure-function relationships of human milk
4 oligosaccharides. *Adv Nutr* 2012; 3:383S-91S.
- 5 19. Katayama T, Sakuma A, Kimura T, Makimura Y, Hiratake J, Sakata K, et al. Molecular
6 cloning and characterization of *Bifidobacterium bifidum* 1,2-a-L-fucosidase (AfcA), a
7 novel inverting glycosidase (glycoside hydrolase family 95). *J Bacteriol* 2004;
8 186:4885-93.
- 9 20. Boren T, Falk P, Roth KA, Larson G, Normark S. Attachment of *Helicobacter pylori* to
10 human gastric epithelium mediated by blood group antigens. *Science* 1993; 262:1892-5.
- 11 21. Ruiz-Palacios GM, Cervantes LE, Ramos P, Chavez-Munguia B, Newburg DS.
12 *Campylobacter jejuni* binds intestinal H(O) antigen (Fuca1, 2Gal b1, 4GlcNAc), and
13 fucosyloligosaccharides of human milk inhibit its binding and infection. *J Biol Chem*
14 2003; 278:14112-20.
- 15 22. Stahl M, Friis LM, Nothaft H, Liu X, Li J, Szymanski CM, et al. L-fucose utilization
16 provides *Campylobacter jejuni* with a competitive advantage. *Proc Natl Acad Sci U S A*
17 2011; 108:7194-9.
- 18 23. Coyne MJ, Reinap B, Lee MM, Comstock LE. Human symbionts use a host-like
19 pathway for surface fucosylation. *Science* 2005; 307:1778-81.
- 20 24. Veith PD, O'Brien-Simpson NM, Tan Y, Djatmiko DC, Dashper SG, Reynolds EC.
21 Outer membrane proteome and antigens of *Tannerella forsythia*. *J Proteome Res* 2009;
22 8:4279-92.
- 23 25. Guillotin L, Lafite P, Daniellou R. Unraveling the substrate recognition mechanism and
24 specificity of the unusual glycosyl hydrolase family 29 BT2192 from *Bacteroides*
25 *thetaiotaomicron*. *Biochemistry* 2014; 53:1447-55.

- 1 26. Berteau O, McCort I, Goasdoué N, Tissot B, Daniel R. Characterization of a new alpha-
2 L-fucosidase isolated from the marine mollusk *Pecten maximus* that catalyzes the
3 hydrolysis of alpha-L-fucose from algal fucoidan (*Ascophyllum nodosum*).
4 *Glycobiology* 2002; 12:273-82.
- 5 27. Rodríguez-Díaz J, Monedero V, Yebra MJ. Utilization of natural fucosylated
6 oligosaccharides by three novel alpha-L-fucosidases from a probiotic *Lactobacillus*
7 *casei* strain. *Appl Environ Microbiol* 2011; 77:703-5.
- 8 28. Winchester B, Barker C, Baines S, Jacob GS, Namgoong SK, Fleet G. Inhibition of alpha-L-
9 fucosidase by derivatives of deoxyfuconojirimycin and deoxymannojirimycin. *Biochem*
10 *J* 1990; 265:277-82.
- 11 29. Hajishengallis G, Lamont RJ. Beyond the red complex and into more complexity: the
12 polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Mol*
13 *Oral Microbiol* 2012; 27:409-19.
- 14 30. Marsh PD. Are dental diseases examples of ecological catastrophes? *Microbiology*
15 2003; 149:279-94.
- 16 31. Marsh PD, Møter A, Devine DA. Dental plaque biofilms: communities, conflict and
17 control. *Periodontol* 2000 2011; 55:16-35.
- 18 32. Tomek MB, Neumann L, Nimeth I, Koerdt A, Andesner P, Messner P, et al. The S-layer
19 proteins of *Tannerella forsythia* are secreted via a type IX secretion system that is
20 decoupled from protein O-glycosylation. *Mol Oral Microbiol* 2014; 29:307-20.
- 21 33. Inagaki S, Onishi S, Kuramitsu HK, Sharma A. *Porphyromonas gingivalis* vesicles
22 enhance attachment, and the leucine-rich repeat BspA protein is required for invasion of
23 epithelial cells by *Tannerella forsythia*. *Infect Immun* 2006; 74:5023-8.
- 24 34. Larsbrink J, Rogers TE, Hemsworth GR, McKee LS, Tausin AS, Spadiut O, et al. A
25 discrete genetic locus confers xyloglucan metabolism in select human gut
26 *Bacteroidetes*. *Nature* 2014; 506:498-502.

- 1 35. Martens EC, Koropatkin NM, Smith TJ, Gordon JI. Complex glycan catabolism by the
2 human gut microbiota: the *Bacteroidetes* Sus-like paradigm. *J Biol Chem* 2009;
3 284:24673-7.
- 4 36. Thompson H, Homer KA, Rao S, Booth V, Hosie AH. An orthologue of *Bacteroides*
5 *fragilis* NanH is the principal sialidase in *Tannerella forsythia*. *J Bacteriol* 2009;
6 191:3623-8.
- 7 37. Cheng HR, Jiang N. Extremely rapid extraction of DNA from bacteria and yeasts.
8 *Biotechnol Lett* 2006; 28:55-9.
- 9 38. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning*. Cold Spring Harbor Laboratory
10 Press, Cold Spring Harbor, New York 1989.
- 11 39. Laemmli UK. Cleavage of structural proteins during the assembly of the head of
12 bacteriophage T4. *Nature* 1970; 227:680-5.
- 13 40. Leonard R, Rendic D, Rabouille C, Wilson IB, Preat T, Altmann F. The *Drosophila*
14 fused lobes gene encodes an *N*-acetylglucosaminidase involved in *N*-glycan processing.
15 *J Biol Chem* 2006; 281:4867-75.
- 16 41. McIlvaine TA. A buffer solution for colorimetric comparison. *J Biol Chem* 1921;
17 49:183-6.
- 18 42. Kurz S, Jin C, Hykollari A, Gregorich D, Giomarelli B, Vasta GR, et al. Hemocytes and
19 plasma of the eastern oyster (*Crassostrea virginica*) display a diverse repertoire of
20 sulfated and blood group A-modified *N*-glycans. *J Biol Chem* 2013; 288:24410-28.
- 21 43. Bradford MM. A rapid and sensitive method for the quantitation of microgram
22 quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;
23 72:248-54.
- 24 44. Honma K, Mishima E, Inagaki S, Sharma A. The OxyR homologue in *Tannerella*
25 *forisythia* regulates expression of oxidative stress responses and biofilm formation.
26 *Microbiology* 2009; 155:1912-22.

1 45. Stafford P, Higham J, Pinnock A, Murdoch C, Douglas CW, Stafford GP, et al.
2 Gingipain-dependent degradation of mammalian target of rapamycin pathway proteins
3 by the periodontal pathogen *Porphyromonas gingivalis* during invasion. Mol Oral
4 Microbiol 2013; 28:366-78.

5

6

7

1 **Figure legends**

2

3 **Figure 1.** SDS PAGE (A.) and Western immunoblot (B.) of total cell extracts from *T.*
4 *forsythia* WT (lane 2) and $\Delta Tffuc1$ strains (lane 3) and of the His₆-tagged rTfFuc1 as purified
5 from *E. coli* (lane 4), used for activity studies and to raise a polyclonal anti-TfFuc1 antiserum.
6 Western immunoblotting using the anti-TfFuc1 antiserum recognized the protein (~51 kDa)
7 specifically in the WT strain (lane 2) and indicated absence of the protein in the $\Delta Tffuc1$ strain
8 (lane 3), proving that the enzyme was effectively knocked-out. In the preparation of rTfFuc 1
9 (B., lane 4), the polyclonal antiserum recognizes also minor contaminating *E. coli* proteins not
10 visible on the SDS-PAGE gel (A., lane 4). Mm; PageRuler Plus prestained protein ladder
11 (Thermo Scientific).

12

13 **Figure 2.** pH profile of rTfFuc1 using 4-nitrophenyl- α -L-fucopyranoside (pNP-fucose) as a
14 substrate. Activity was measured as the increase in Abs₄₀₅ due to the released 4-nitrophenol
15 product. Citrate/phosphate buffer (0.1 M) was used to assay the pH range from 3-8, 50 mM
16 glycine buffer was used for the pH range from 8-10.25.

17

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

21

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

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

6

7 **Figure 6.** rTfFuc1 was incubated with mucin from bovine submaxillary glands and the release
8 of fucose was measured with the K-FUCOSE kit. When incubations were performed in
9 conjunction with the rNanH sialidase, a slow steady increase in the Abs₃₄₀ was observed. The
10 activity was calculated over a period of 300 s where the data points fitted a linear regression
11 with an r^2 of 0.98. No activity could be detected, when rTfFuc1 was incubated alone with the
12 mucin. The Δ Abs₃₄₀ lead to an irregular data set with a very low r^2 value of 0.4.

13

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

23

1 **Table 1.** rFuc1 activity on 4-nitrophenyl- α -L-fucopyranoside (pNP-fucose). Catalytic
 2 constants K_M and V_{max} and the inhibitory effect of DFJ and L-fucose were measured using the
 3 colorimetric substrate pNP-fucose within a concentration range from 0.01 to 50 mM at 22°C
 4 in glycine buffer at pH 9.0

5

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

6

7 *4-nitrophenyl- α -L-fucopyranoside

8 ** μ mol/min/mg of enzyme

9 ***deoxyfuconojirimycin

10

11

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	-

* μ mol/min/mg of enzyme

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant characteristic(s)	Source
<i>Escherichia coli</i> DH5 α	F ⁻ ϕ 80d <i>lacZ</i> M15 (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1</i> <i>hsdR17</i> (rK ⁻ mK ⁻) <i>phoA supE44 thi-1 gyrA96 relA1⁻</i>	Invitrogen
<i>Escherichia coli</i> BL21 (DE)	F, <i>ompT</i> , <i>hsdS</i> (<i>rB⁻mB⁻</i>), <i>gal</i> , <i>dcm</i> (DE3)	Invitrogen
<i>Tannerella forsythia</i> ATCC 43037	Wild-type isolate	American Type Culture Collection, USA
<i>T. forsythia</i> Δ <i>Tffuc1</i>	<i>T. forsythia</i> knockout of the <i>Tffuc1</i> gene; Erm ^r	This study
pET-22b (+)	Expression vector with a His ₆ -tag, Amp ^r	Novagen
pJET1.2- <i>Tffuc1</i> _ko	pJET1.2 carrying the <i>Tffuc1_ermF-AM</i> knockout cassette	This study

Table 4. Oligonucleotide primers used for PCR amplification reactions^a

Primers	Sequence (5' → 3') ^a
1	gcgg <u>CATATG</u> AAAACAAGAACATTACTTCTTTGTG
2	gcta <u>CTCGAGG</u> TTTAGAGGCAATTCATTGGCAAATG
3	GACCAAGCTGCAGGCCATCATCGATGTGCTCAAC
A	<i>GAAGCTATCGGGGGTACCTCCCCGGG</i> -AGAATAATTTTTGTTTATTACTAAAAATAACG
5	<i>GCTTCGGGGATCCTCTAGCCCCGGG</i> -CAGAAATATCTTTATGAAACATCCTATTGATGGGGTG
6	GCTCAGCCAGCCGATAGTTACTTTTTTCGTTATGTGTTCCC
7 ^b	CGTTATTTTTTAGTAATAAACAAAATTATTCT-<u>CCCGGGGAGGTACCCCGATAGCTTC</u>
8 ^b	CACCCCATCAATAGGATGTTTCATAAAGATATTTCTG-<u>CCCGGGGGCTAGAGGATCCCGAAGC</u>
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

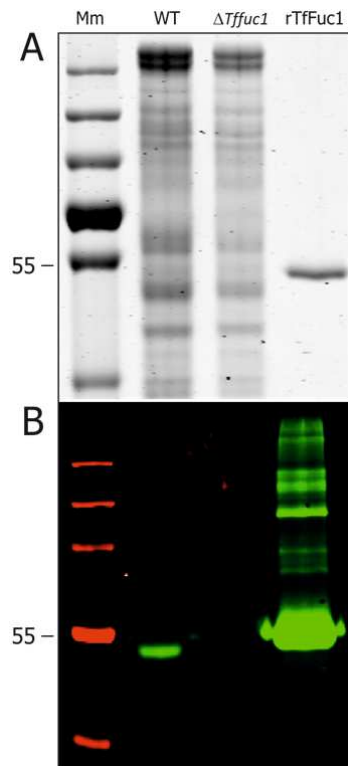


Figure 1. of Megson *et al.*

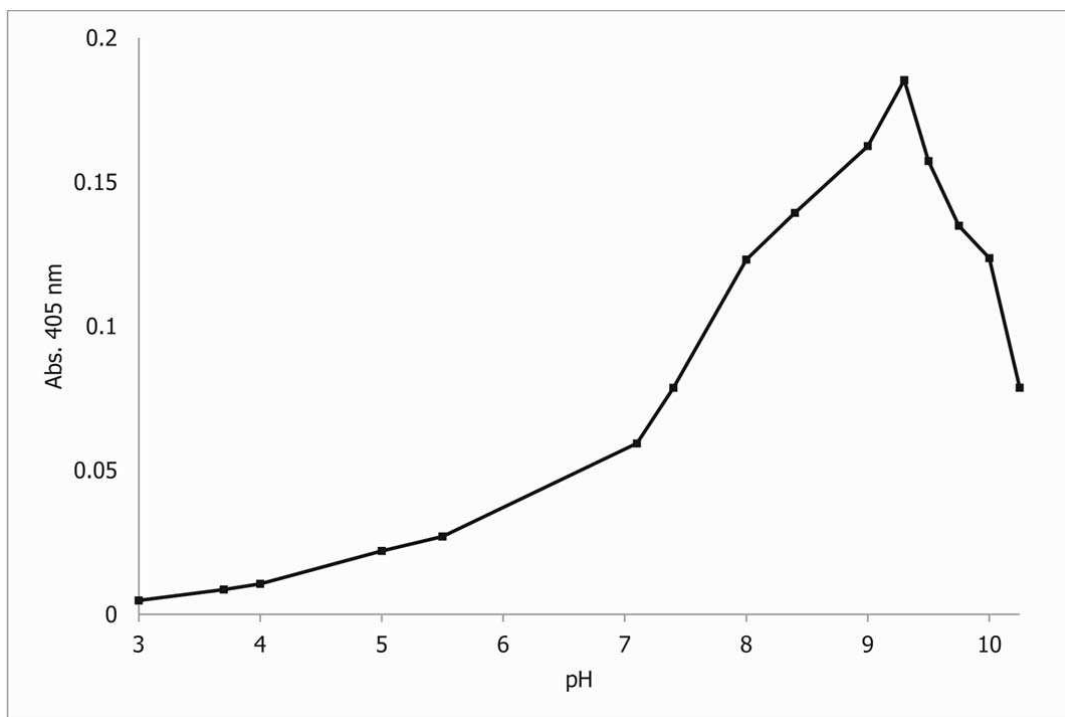


Figure 2. of Megson *et al.*

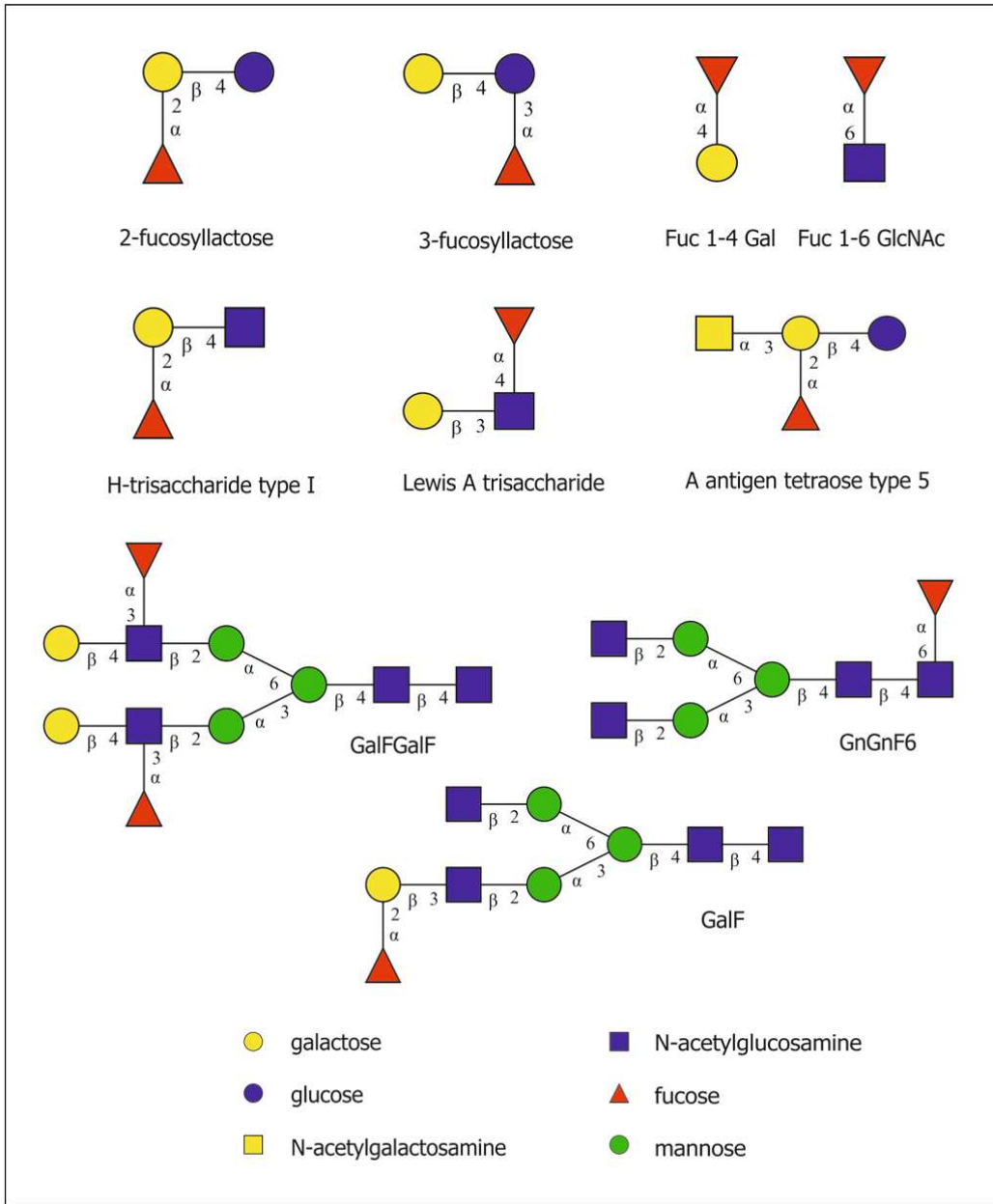


Figure 3. of Megson *et al.*

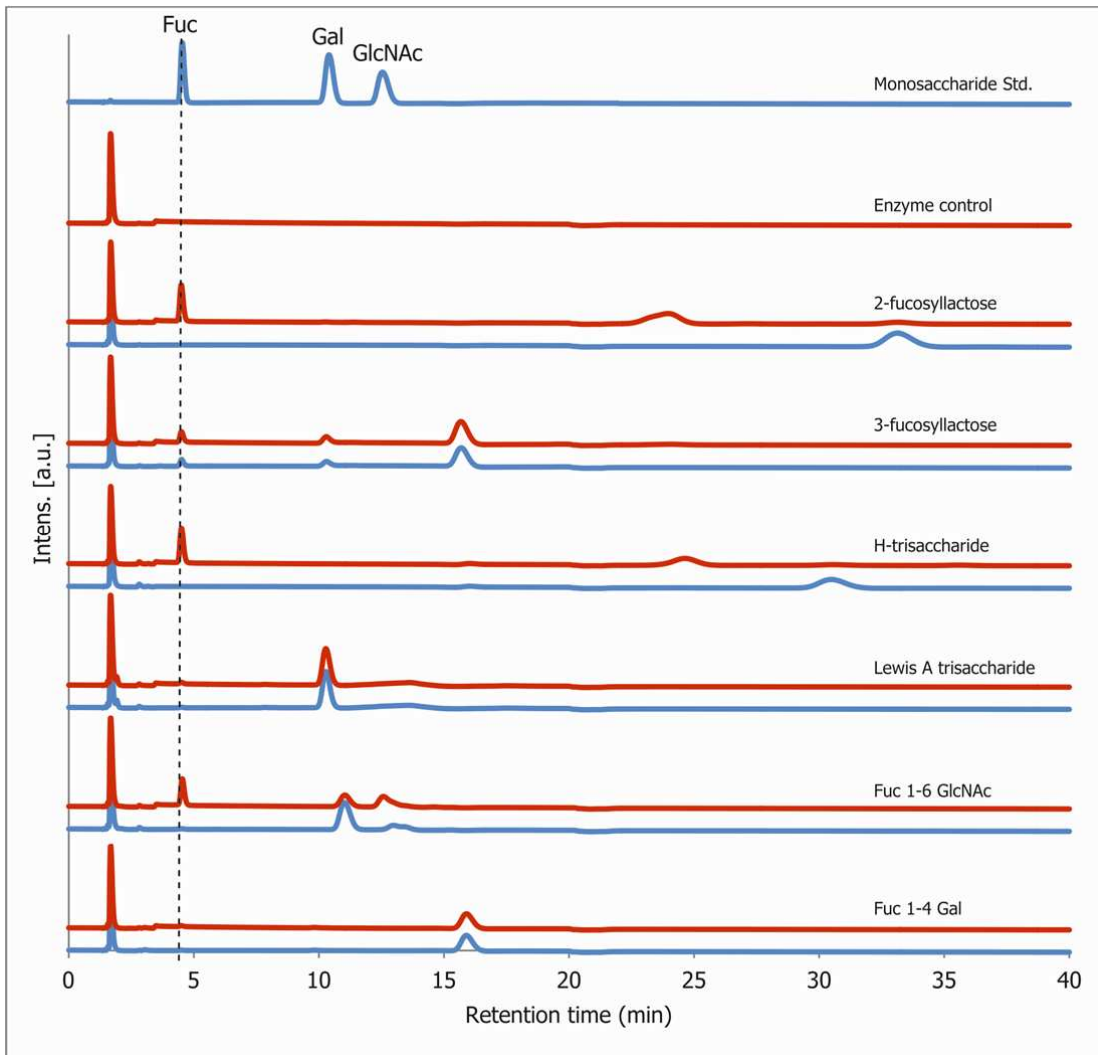


Figure 4. of Megson *et al.*

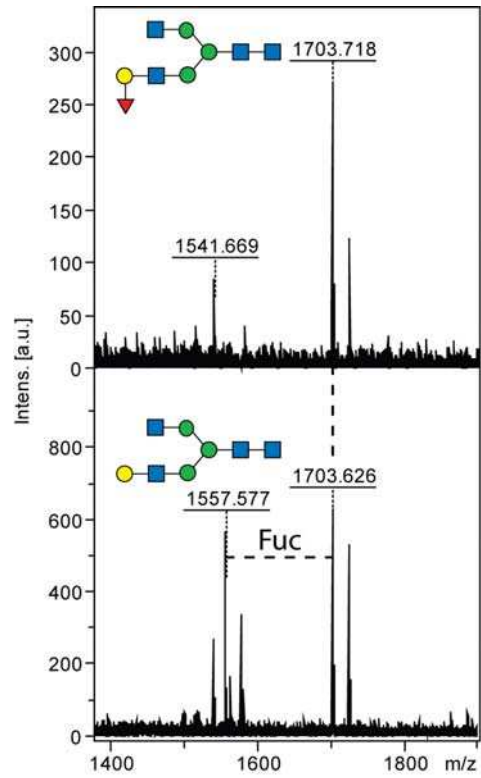


Figure 5. of Megson *et al.*

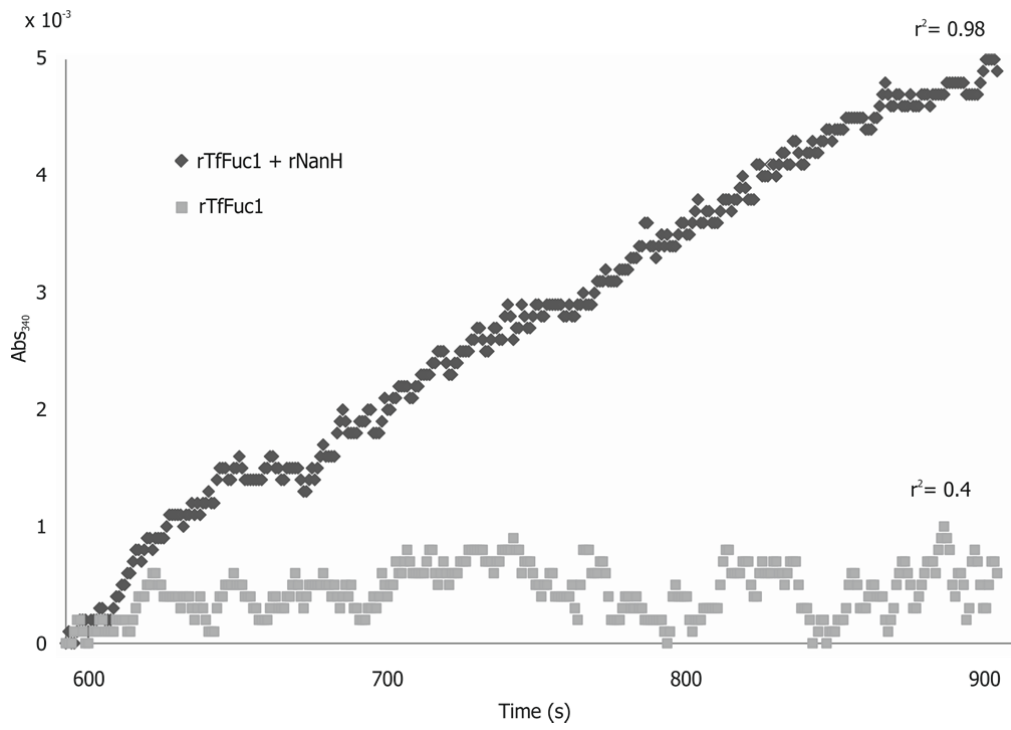


Figure 6. of Megson *et al.*

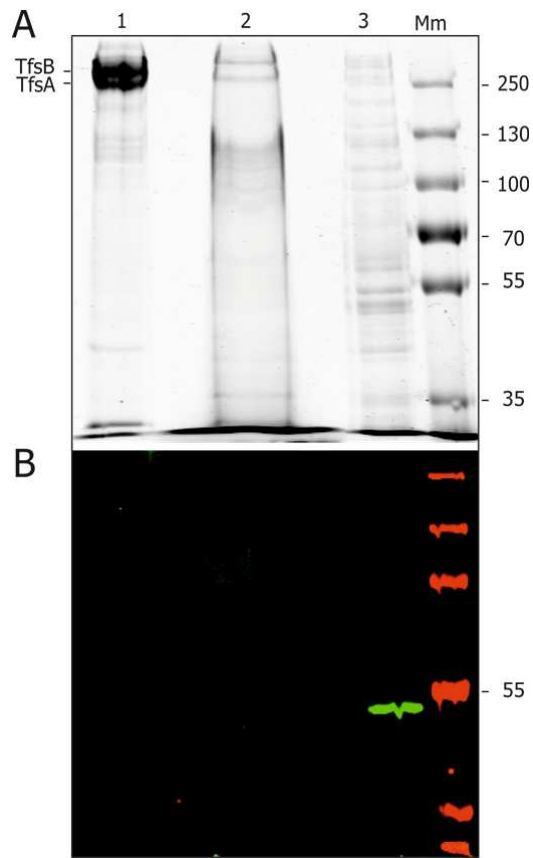


Figure 7. of Megson *et al.*

Table 1. rFuc1 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 (\pm 0.2)	20.4 (\pm 0.8)
pNp-fucose	0.1 μ M DFJ***	28.3 (\pm 3.7)	28.1 (\pm 2.4)
pNp-fucose	0.25 mM Fucose	16.5 (\pm 4.7)	28.6 (\pm 4.4)

*4-nitrophenyl- α -L-fucopyranoside

** μ mol/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	-

* μ mol/min/mg of enzyme

Table 3. Bacterial strains and plasmids used in this study

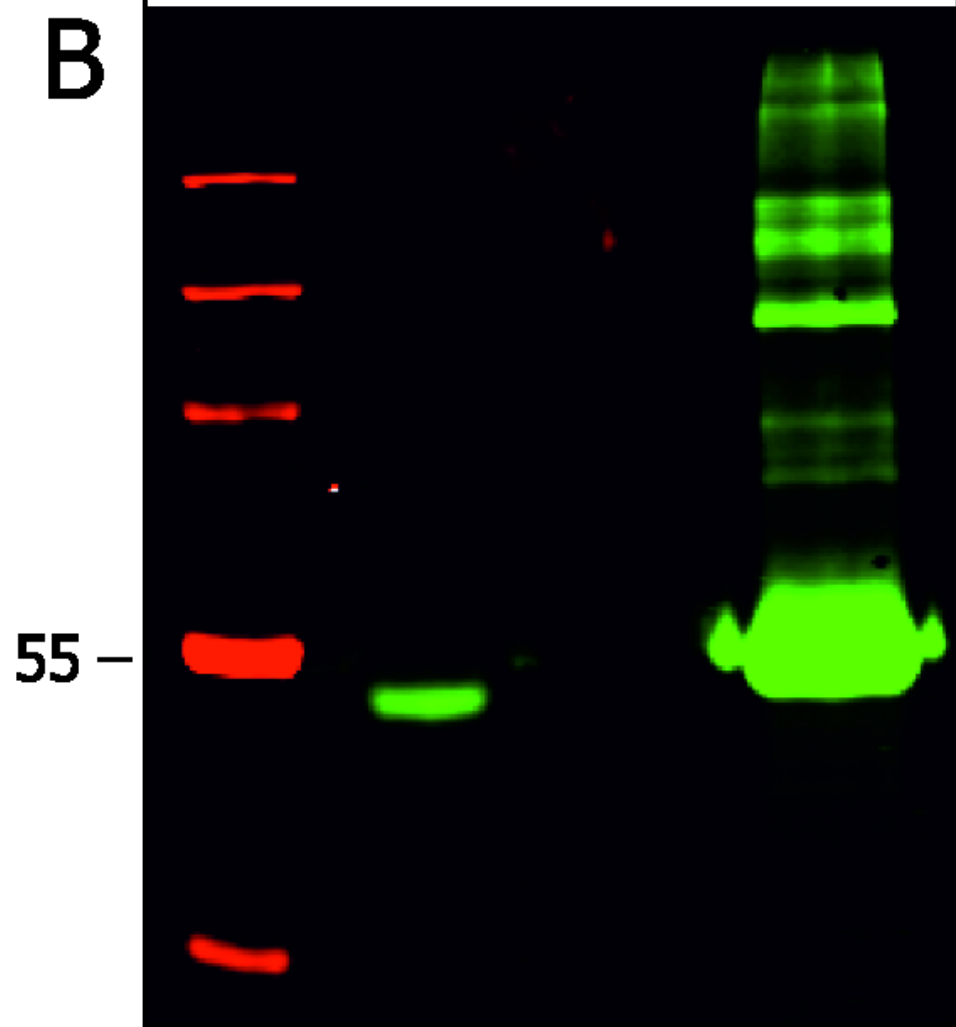
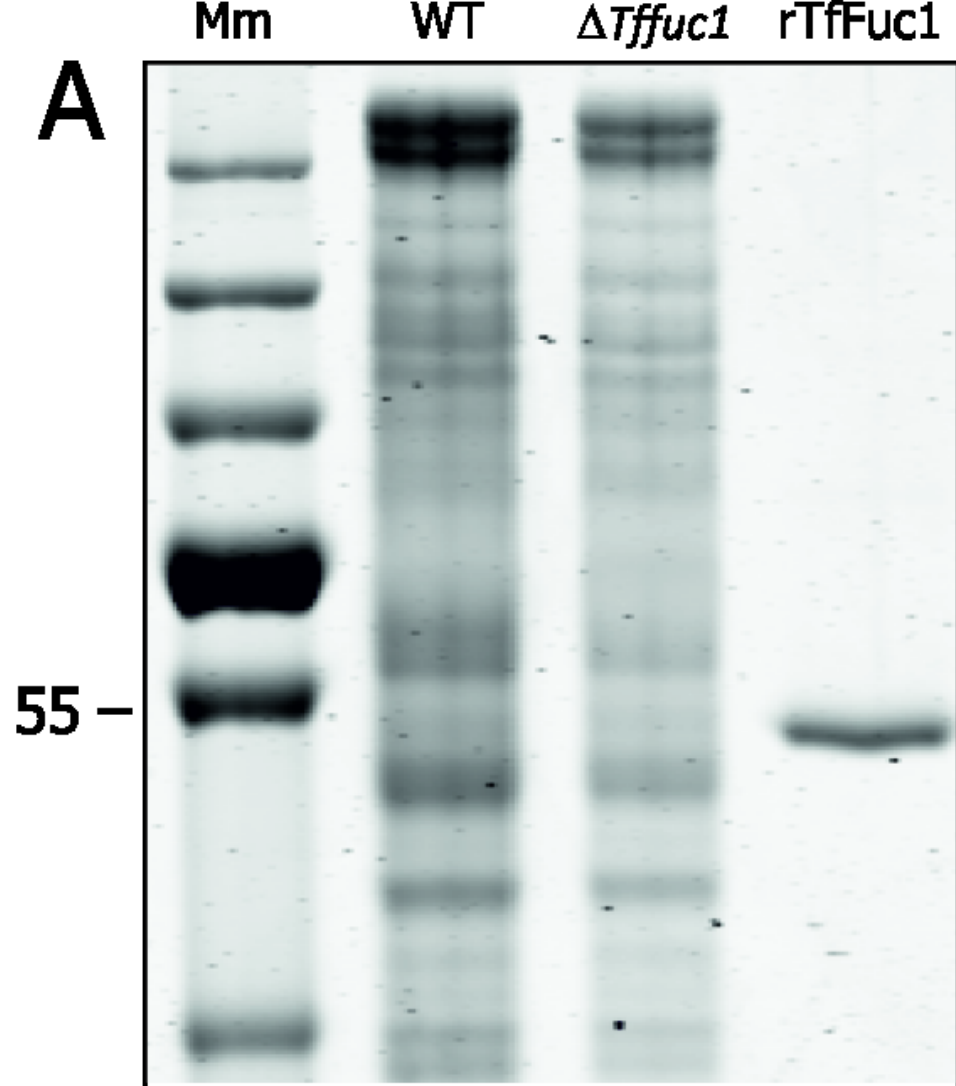
Strain or plasmid	Genotype and/or relevant characteristic(s)	Source
<i>Escherichia coli</i> DH5 α	F ⁻ ϕ 80d <i>lacZ</i> M15 (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1</i> <i>hsdR17</i> (rK ⁻ mK ⁻) <i>phoA supE44 thi-1 gyrA96 relA1⁻</i>	Invitrogen
<i>Escherichia coli</i> BL21 (DE)	F ⁻ , <i>ompT</i> , <i>hsdS</i> (<i>rB⁻mB⁻</i>), <i>gal</i> , <i>dcm</i> (DE3)	Invitrogen
<i>Tannerella forsythia</i> ATCC 43037	Wild-type isolate	American Type Culture Collection, USA
<i>T. forsythia</i> Δ <i>Tffuc1</i>	<i>T. forsythia</i> knockout of the <i>Tffuc1</i> gene; Erm ^r	This study
pET-22b (+)	Expression vector with a His ₆ -tag, Amp ^r	Novagen
pJET1.2- <i>Tffuc1_</i> ko	pJET1.2 carrying the <i>Tffuc1_ermF-AM</i> knockout cassette	This study

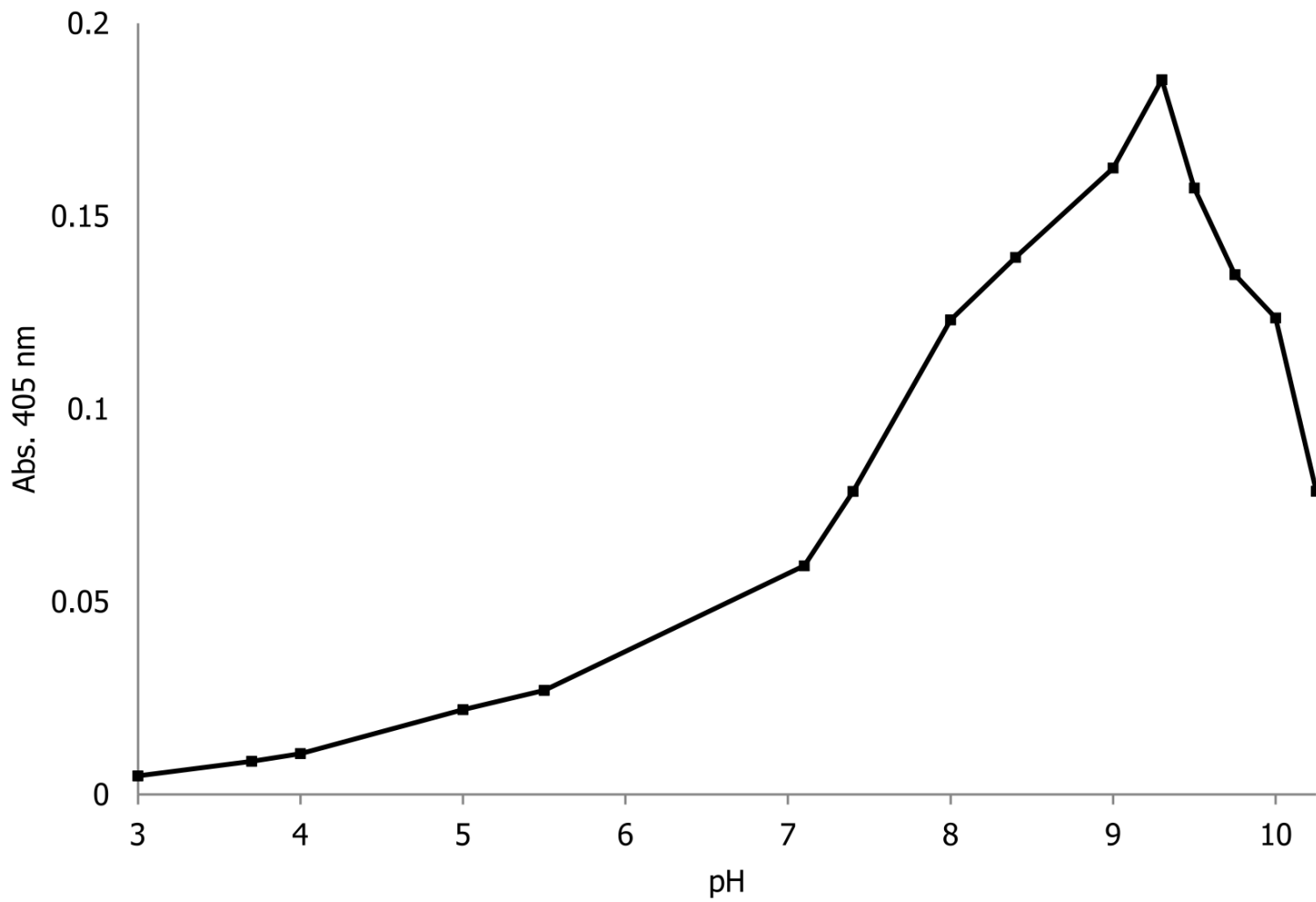
Table 4. Oligonucleotide primers used for PCR amplification reactions^a

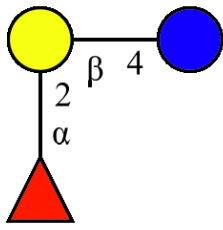
Primers	Sequence (5' → 3') ^a
1	gcgg <u>CATATG</u> AAAACAAGAACATTACTTCTTTGTG
2	gcta <u>CTCGAGG</u> TTTAGAGGCAATTCATTGGCAAATG
3	GACCAAGCTGCAGGCCATCATCGATGTGCTCAAC
4	<i>GAAGCTATCGGGGGTACCTCCCCGGG</i> -AGAATAATTTTTGTTTATTACTAAAAATAACG
5	<i>GCTTCGGGGATCCTCTAGCCCCGGG</i> -CAGAAATATCTTTATGAAACATCCTATTGATGGGGTG
6	GCTCAGCCAGCCGATAGTTACTTTTTTCGTTATGTGTTCCC
7 ^b	CGTTATTTTTTAGTAATAAACAAAATTATTCT-<u>CCCGGGGGAGGTACCCCGATAGCTTC</u>
8 ^b	CACCCCATCAATAGGATGTTTCATAAAGATATTTCTG-<u>CCCGGGGGCTAGAGGATCCCGAAGC</u>
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.

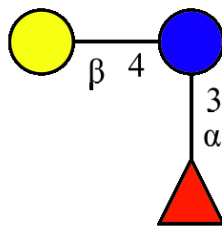
b. Primer sequences were taken from Honma et al.¹¹



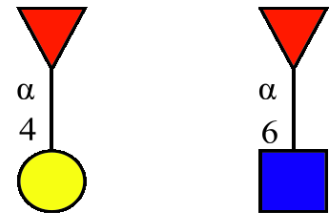




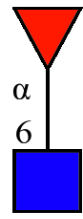
2-fucosyllactose



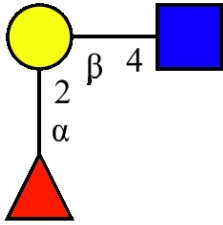
3-fucosyllactose



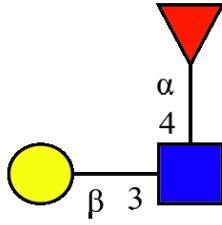
Fuc 1-4 Gal



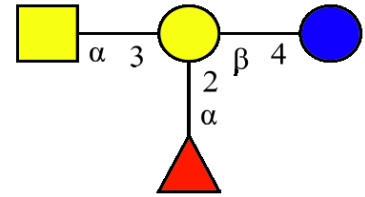
Fuc 1-6 GlcNAc



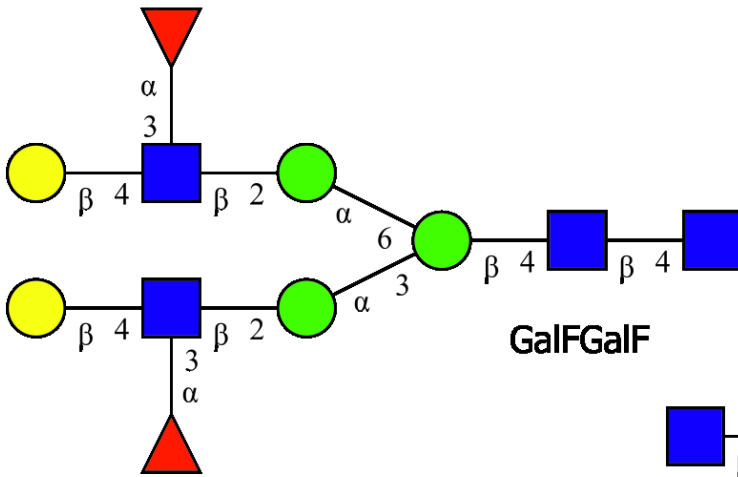
H-trisaccharide type I



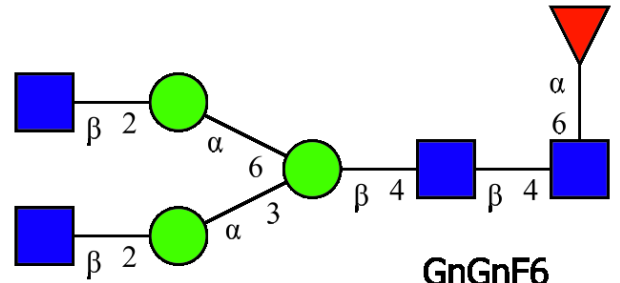
Lewis A trisaccharide



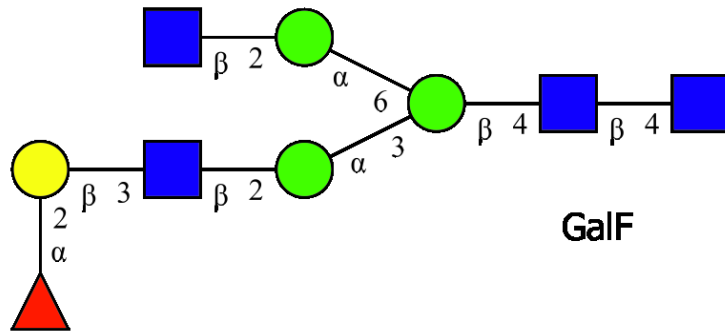
A antigen tetraose type 5



GalFGalF



GnGnF6



GalF

● galactose

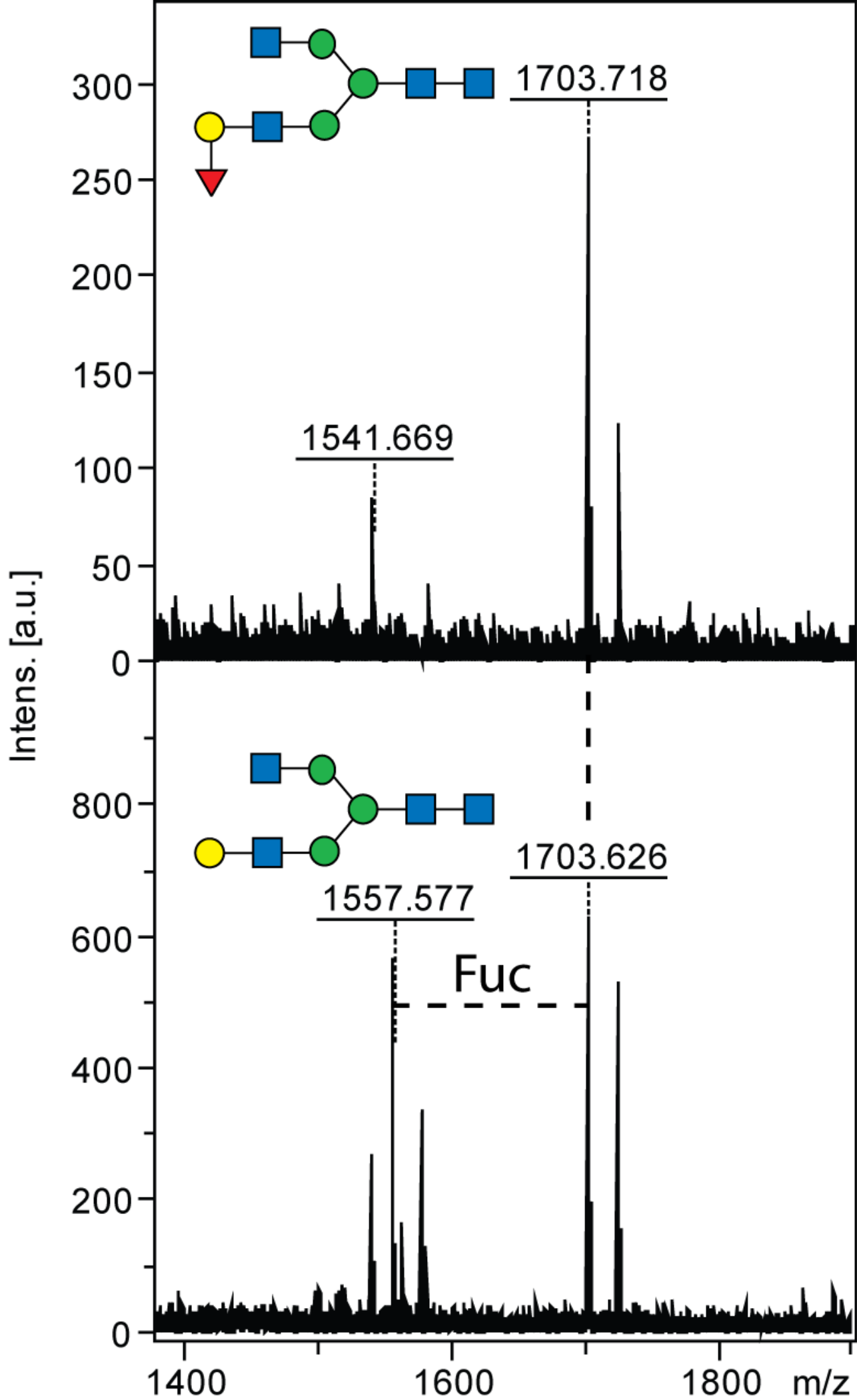
● glucose

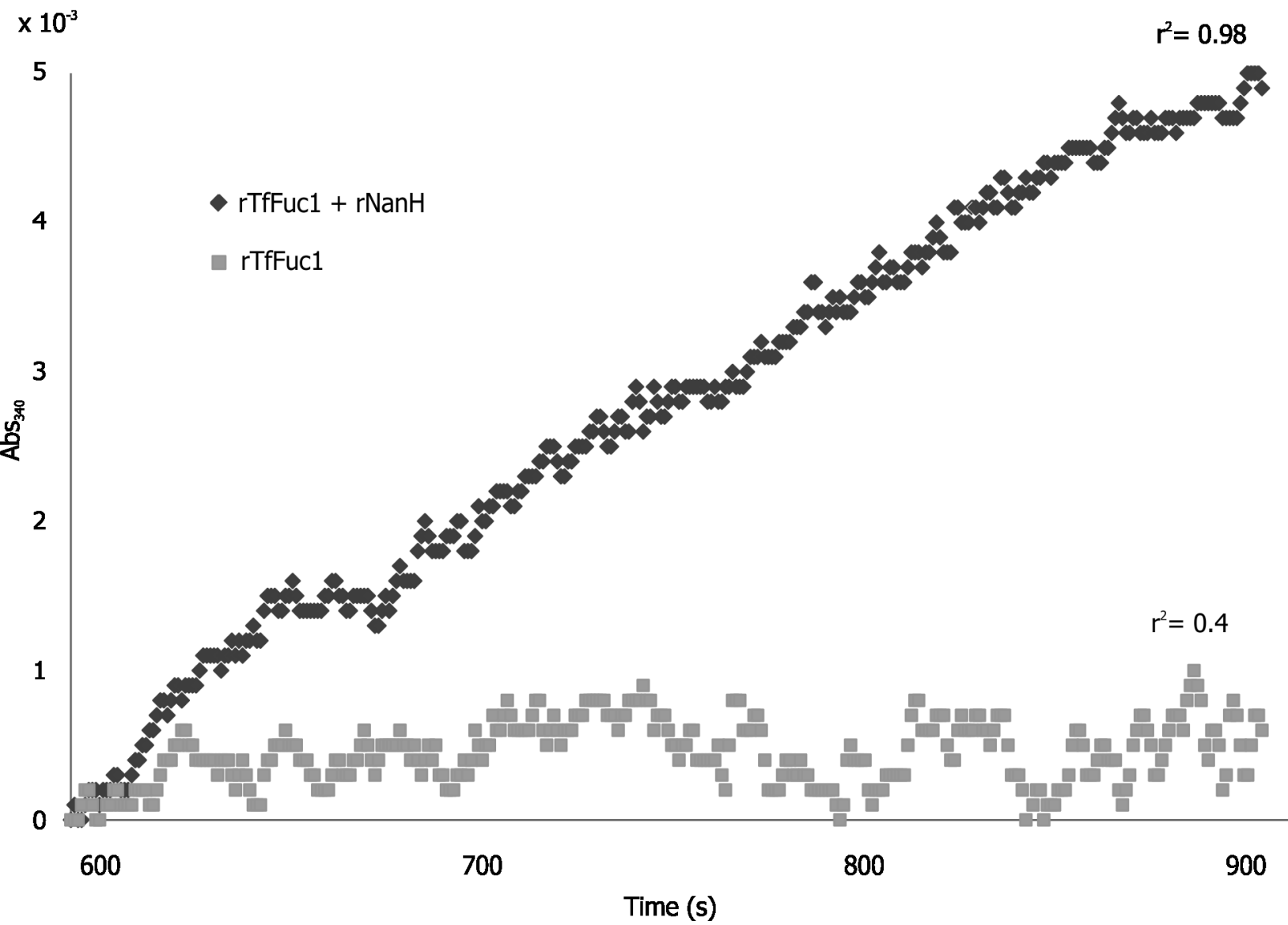
■ N-acetylgalactosamine

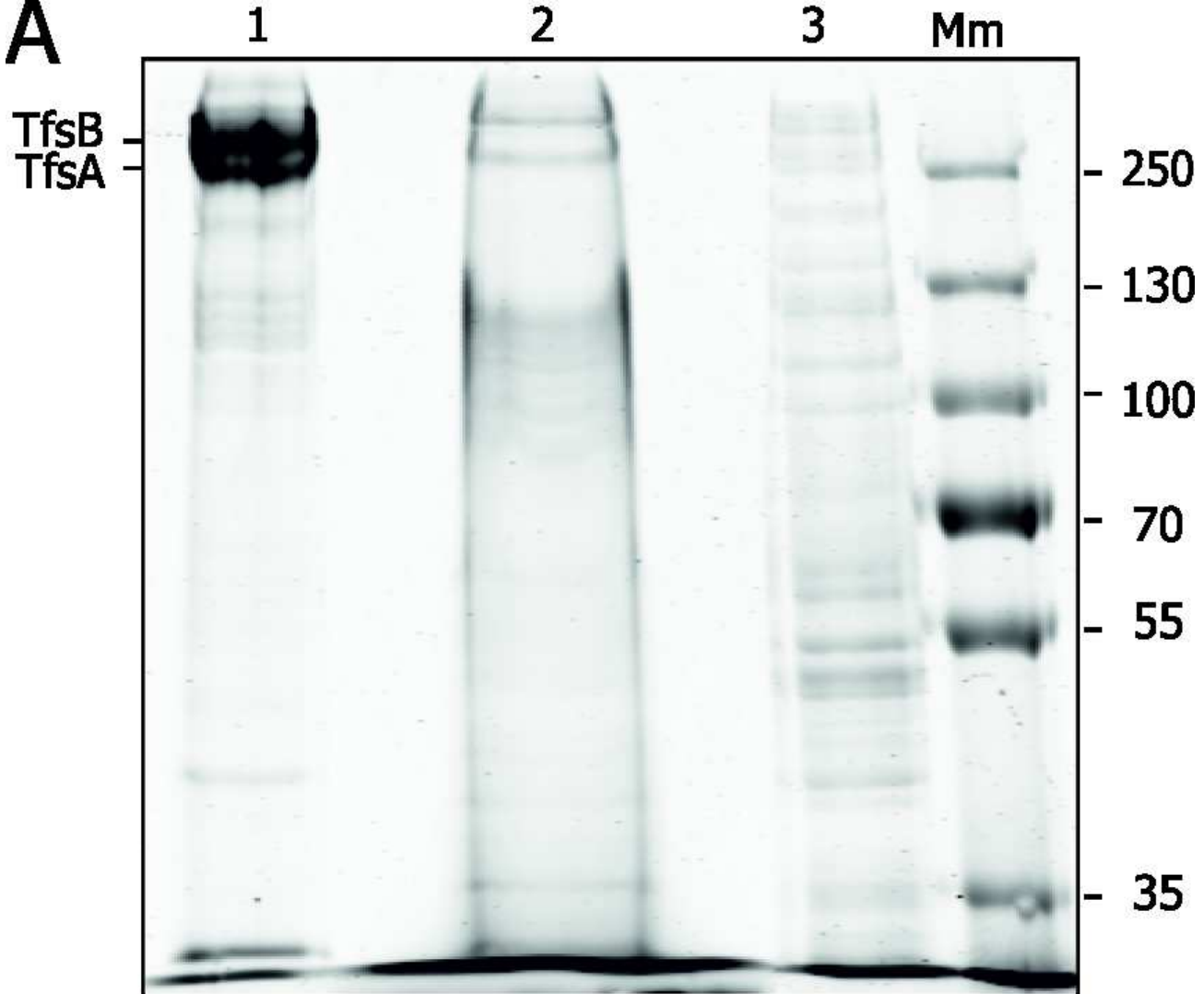
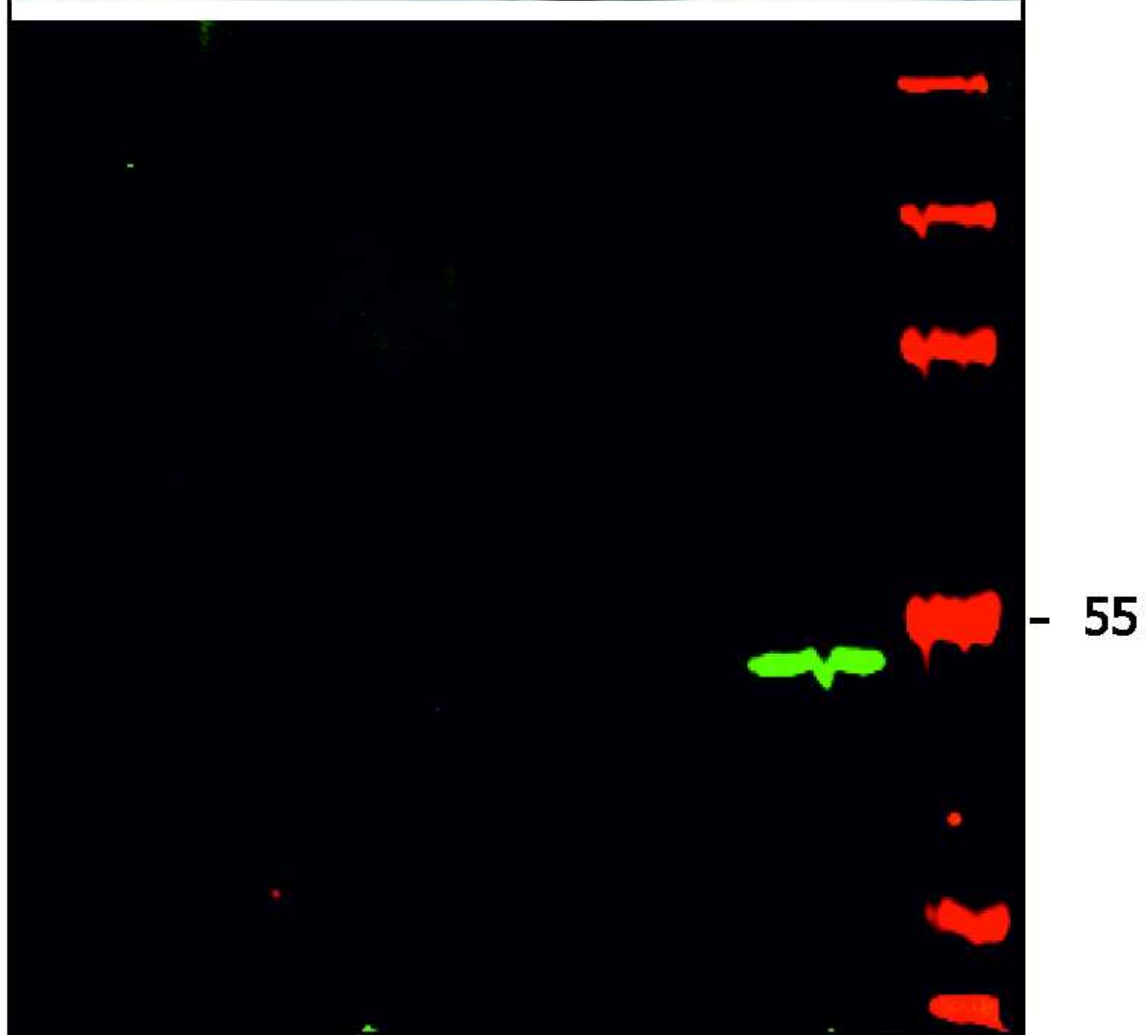
■ N-acetylglucosamine

▲ fucose

● mannose





A**B**

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% CO₂. H357 cells were grown to near-confluence (90 to 95%) for the assays.

For both attachment and invasion assays, *T. forsythia* WT and Δ Tffuc1 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 μ g/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 Δ Tffuc1 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 μ l/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 OD₆₀₀ ~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 $\mu\text{g/ml}$) and gentamycin/erythromycin (50 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$) 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 $\mu\text{g/ml}$ 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 ($\text{OD}_{600} \sim 1.0$), 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 Δ Tffuc1 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 OD₆₀₀ 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 Δ Tffuc1 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 Δ fuc mutant was 1.2-fold that of the WT. In the case of half-strength medium, the difference became more prominent as Δ fuc 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.

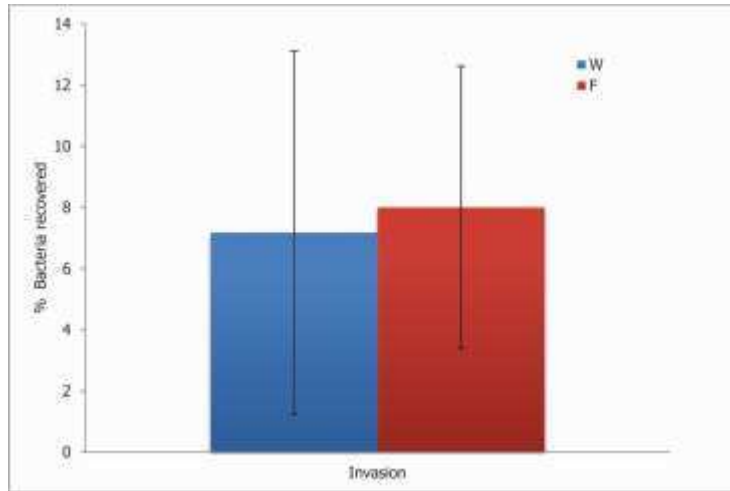


Figure S1. Cell invasion assays. The effect of the Tffuc1 mutation on the ability of *T. forsythia* to interact and invade human oral epithelial cells was tested on the oral epithelial cell line H357. The combined results from three experimental repeats are shown as a percentage of the bacterial cells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

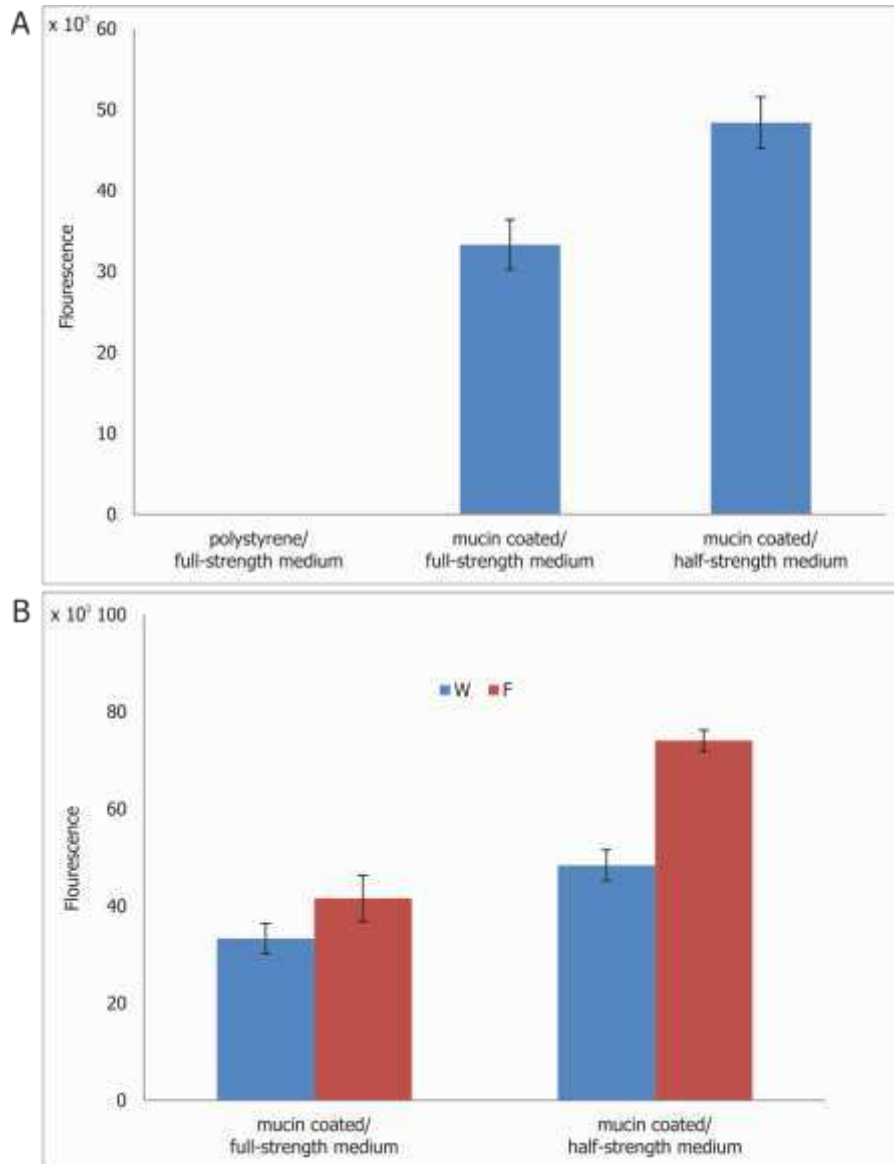


Figure S2. Microtiter assays of Hoechst-stained biofilms. **A.** The WT strain increased its biofilm formation on a mucin-coated surface as the strength of the medium was decreased to half that of the original broth. **B.** The difference in biofilm formation between the WT and the $\Delta Tffuc1$ depends largely on the strength of the medium. W, *T. forsythia* wild-type; F, *T. forsythia* $\Delta Tffuc1$.

