Outer membrane vesicles of *Tannerella forsythia*: biogenesis, composition, and virulence

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SUMMARY

*Tannerella forsythia* is the only ‘red-complex’ bacterium covered by an S-layer, which has been shown to affect virulence. Here, outer membrane vesicles (OMVs) enriched with putative glycoproteins are described as a new addition to the virulence repertoire of *T. forsythia*. Investigations of this bacterium are hampered by its fastidious growth requirements and the recently discovered mismatch of the available genome sequence (92A2 = ATCC BAA-2717) and the widely used *T. forsythia* strain (ATCC 43037). *T. forsythia* was grown anaerobically in serum-free medium and biogenesis of OMVs was analyzed by electron and atomic force microscopy.

This revealed OMVs with a mean diameter of ~100 nm budding off from the outer membrane while retaining the S-layer. An LC-ESI-TOF/TOF proteomic analysis of OMVs from three independent biological replicates identified 175 proteins. Of these, 14 exhibited a C-terminal outer membrane translocation signal that directs them to the cell/vesicle surface, 61 and 53 were localized to the outer membrane and periplasm, respectively, 22 were predicted to be extracellular, and 39 to originate from the cytoplasm. Eighty proteins contained the *Bacteroidales O*-glycosylation motif, 18 of which were confirmed as glycoproteins. Release of proinflammatory mediators from the human monocytic cell line U937 and periodontal ligament fibroblasts upon stimulation with OMVs followed a concentration-dependent increase that was more pronounced in the presence of soluble CD14 in conditioned media. The inflammatory response was significantly...
higher than that caused by whole \textit{T. forsythia} cells. Our study represents the first characterization of \textit{T. forsythia} OMVs, their proteomic composition and immunogenic potential.

**Keywords**

atomic force microscopy; electron microscopy; inflammatory response; outer membrane vesicles; periodontal pathogen; \textit{Tannerella forsythia} strain ATCC 43037 \textit{versus} strain 92A2

**INTRODUCTION**

\textit{Tannerella forsythia}, \textit{Porphyromonas gingivalis} and \textit{Treponema denticola} are Gram-negative anaerobes that have been identified as major periodontal pathogens (Socransky \textit{et al.}, 1998). Together, they constitute the so-called ‘red-complex’ consortium that colonizes dental plaque biofilms (Holt & Ebersole, 2005) and is strongly associated with the clinical measures of periodontitis, a multifactorial, inflammatory disease of global importance (Darveau, 2010). According to a more recent model of pathogenesis, periodontitis is initiated by a synergistic and dysbiotic microbial community rather than by select periodontal pathogens, such as the ‘red complex’ (Hajishengallis & Lamont, 2012). In this polymicrobial synergy, different members or specific gene combinations within the community fulfill distinct roles that converge to shape and stabilize a disease-provoking microbiota.

Apart from oral health issues, emerging evidence shows a relationship between periodontitis, cardiovascular disease, rheumatoid arthritis and other systemic chronic diseases (Cullinan \textit{et al.}, 2009; Koziel \textit{et al.}, 2014). For the development of new therapeutic strategies to combat periodontitis, a molecular understanding of the mechanisms governing bacterial virulence is required. Apart from distinct virulence factors that have been identified for the ‘red-complex’ bacteria (O’Brien-Simpson \textit{et al.}, 2004), outer membrane vesicles (OMVs), which are also known as integral parts of biofilm matrices (Flemming \textit{et al.}, 2007), are emerging as ‘bacterial warfare’ agents in the pathogenesis of periodontitis.

Generally, OMVs are natural secretion products of Gram-negative bacteria, with an increasing number of pathogens being reported to release OMVs (Unal \textit{et al.}, 2011; Schertzer & Whiteley, 2013). They are small, spherical particles, usually 20–250 nm in diameter, and secreted throughout the bacterial life cycle and in a wide range of environmental conditions. Although the mechanism of OMV biogenesis is still poorly understood, studies so far point towards a highly regulated process that is most likely essential to the cell. Current models suggest that vesiculation occurs by budding off from the outer membrane (OM) at sites where lipoprotein links between the OM and the peptidoglycan are lost (Kulp & Kuehn, 2010). OMVs are characterized by selective enrichment or exclusion of specific cargo (Haurat \textit{et al.}, 2011); containing, apart from OM-derived components such as lipopolysaccharide (LPS), phospholipids and OM proteins, also periplasmic constituents. OMVs are gaining increasing attention as a major mechanism by which pathogens attack and interact with host cells. As such, they have been shown to play roles in establishing colonization, carrying and transmitting virulence factors into host cells, and modulating host defense and response (Ellis & Kuehn, 2010).
Regarding the ‘red-complex’ consortium, OMVs have so far only been described in detail for *P. gingivalis* (Veith et al., 2014), which is generally the best investigated ‘red-complex’ bacterium. *P. gingivalis* OMVs are found in gingival tissues at diseased sites in chronic periodontitis but not at healthy sites (O’Brien-Simpson et al., 2009) and are recognized as important virulence factors that are produced when *P. gingivalis* is part of a polymicrobial biofilm (Zhu et al., 2013). The *P. gingivalis* OMVs can invade host epithelial cells via an endocytic pathway (Furuta et al., 2009) and impair cellular functions by the gingipain-exerted degradation of important receptor proteins (Veillard et al., 2012; Wilensky et al., 2015). In the context of the ‘red complex’, a synergistic effect of *P. gingivalis* OMVs has become obvious through their enhancement of *T. forsythia* attachment to epithelial cells (Inagaki et al., 2006). A recent study supports an interdependence between *P. gingivalis* virulence factors and *T. forsythia*, revealing that *P. gingivalis* gingipains influence the composition of polymicrobial biofilms (Bao et al., 2014).

For *T. forsythia*, the identification of new virulence factors (Sharma, 2010) is hampered by its fastidious growth requirements and the recently discovered mismatch of the available genome sequence (92A2=ATCC BAA-2717) and the deposited *T. forsythia* strain (ATCC 43037) (Friedrich et al., 2015). So far, only a few putative virulence factors of *T. forsythia* have been identified. These include trypsin-like (Amano et al., 2014) and PrtH (Saito et al., 1997) proteases, the sialidases SiaH (Horstman & Kuehn, 2000) and NanH (Thompson et al., 2009; Stafford et al., 2012), apoptosis-inducing activity (Evans et al., 2012), α-β-glucosidase and N-acetyl-β-glucosaminidase (Hughes et al., 2003), a hemagglutinin (Bomberger et al., 2009), methylglyoxal (Forsberg et al., 1981), a leucine-rich repeat cell surface-associated and secreted protein BspA (Sharma et al., 1998), the KLIKK proteases (Ksiazek et al., 2015b) such as karilysin (Karim et al., 2010; Koziel et al., 2010) and mirolase (Ksiazek et al., 2015a) as well as the S-layer (Sabet et al., 2003). The latter belong to the major class of proteins carrying a C-terminal OM translocation signal known as CTD (C-terminal domain) (Lee et al., 2006; Sato et al., 2013; Narita et al., 2014; Tomek et al., 2014). Similarly to *P. gingivalis* and *Treponema denticola* (Dashper et al., 2011), *T. forsythia* secretes large amounts of CTD proteins by directing them to a type IX secretion system (T9SS), the presence of which has been demonstrated in *T. forsythia* only recently (Narita et al., 2014; Tomek et al., 2014). Some of these CTD proteins have been recognized as virulence factors (Veith et al., 2009b; Sharma, 2010). In this context it is interesting to note that both of the S-layer proteins (TfsA and TfsB) as well as BspA are glycoproteins (Veith et al., 2009b). The S-layer glycoproteins (TfsA–GP, TfsB–GP) are intercalated on the bacterial cell surface to form a so far unique two-dimensional crystalline monolayer (Sekot et al., 2012). For TfsA and TfsB it was shown experimentally that they are modified by the general protein O-glycosylation system of *T. forsythia* by the addition of multiple copies of a complex oligosaccharide (Posch et al., 2011). A trisaccharide branch of this oligosaccharide, composed of two N-acetylmannosaminuronic acid residues and one modified nonulosonic acid, was shown to modulate dendritic cell effector functions to suppress T-helper 17 responses, thereby ensuring the persistence of the pathogen in the host (Settem et al., 2013). It is evident that several other *T. forsythia* proteins are targeted by the general O-glycosylation system (Posch et al., 2011), with BspA likely to be among them.
To contribute to the understanding of virulence mechanisms of *T. forsythia*, this study was designed to shed light on the bacterium’s OMVs and their putative role in shuttling virulence factors to reach distant cells in a concentrated, protected and targeted form. Here, (i) the biogenesis and morphology of *T. forsythia* OMVs were visualized by electron microscopy using ultrathin-sectioned and negatively stained bacterial cells as well as isolated OMVs; the native situation of vesiculation was investigated by atomic force microscopy (AFM); (ii) a compositional analysis of the OMVs was performed; specifically, the content of S-layer glycoproteins and LPS was determined; (iii) a shotgun proteomics approach was used to identify OMV proteins followed by categorizing them according to their predicted cellular location and their CTD OM translocation signal, with a focus on putative glycoproteins; and (iv) the release of proinflammatory mediators potentially relevant to the development of periodontitis [tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-8, monocyte chemoattractant protein 1 (MCP-1)] from human macrophages and periodontal ligament fibroblasts was determined in comparison to whole *T. forsythia* cells. Our data support the virulent character of *T. forsythia* OMVs and indicate that they are enriched in putative glycoproteins, strengthening the importance of the interplay between glycobiology and virulence in this periodontal pathogen.

Considering that all published data on *T. forsythia* ATCC 43037 genes and proteins, so far, were assigned to the genome sequence of the wrong strain (92A2=ATCC BAA-2717), it is another aim of this study to perform a profound comparison of our OMV proteome data, based on the correct, recently sequenced genome of *T. forsythia* ATCC 43037 (Friedrich et al., 2015), to the published OM proteome data of *T. forsythia* (Veith et al., 2009a). This should facilitate the identification of candidates for developing diagnostics and therapeutics against *T. forsythia* infection.

**METHODS**

**Bacterial strain and growth conditions**

*Tannerella forsythia* wild-type strain ATCC 43037 was purchased from ATCC (Manassas, VA) and grown anaerobically at 37°C for up to 7 days in 37 g l⁻¹ of brain–heart infusion broth (Oxoid, Basingstoke, UK) supplemented with 10 g l⁻¹ yeast extract (Oxoid), 1 g l⁻¹ L-cysteine (Sigma, Vienna, Austria), 2 μg ml⁻¹ menadione (Sigma), 5 μg ml⁻¹ hemin (Sigma) and 20 μg ml⁻¹ N-acetylmuramic acid (Carbosynth, Compton, UK). To avoid interference of serum proteins with immunological and proteomic analyses, no serum was added to the growth medium.

**Preparation of OMVs**

The preparation of OMVs essentially followed a published protocol (Kadurugamuwa & Beveridge, 1995). Briefly, cells from a 200-ml *T. forsythia* culture at an optical density at 600 nm (OD₆₀₀) of ~1.0 were pelleted (20 min, 10,000 g, 4°C) and the supernatant was sequentially filtered through a 0.45-μm and a 0.22-μm pore-size PVDF membrane (Stericup-GV, Millipore, Billerica, MA) to remove residual cell debris. Subsequently, the filtrate was subjected to ultracentrifugation (2 h, 100,000 g, 4°C) to recover OMVs; the pellet was washed twice with sterile phosphate-buffered saline (PBS), resuspended in 25 ml of PBS
and ultracentrifuged again. For immunological studies, the pellet was finally resuspended in 2 ml of sterile PBS and stored in aliquots at −20°C. For proteomic analyses, the OMVs were resuspended in 10 μl Laemmli buffer (Laemmli, 1970) per mg of pellet.

**Microscopy**

One milliliter of *T. forsythia* ATCC 43037 culture was centrifuged (5 min, 4500 g, 4°C), washed twice with PBS and resuspended in 500 μl of PBS. Ten-microliter aliquots of the concentrated sample were used for microscopic analyses. Transmission electron microscopy (TEM) of negatively stained and ultrathin-sectioned preparations of *T. forsythia* cells as well as of isolated *T. forsythia* OMVs was performed on a Tecnai G2 20 Twin microscope (FEI, Eindhoven, the Netherlands) operating at 120 kV, as described previously (Messner *et al.*, 1986; Sekot *et al.*, 2012). For non-invasive in vitro imaging by AFM using a Nanoscope III multimode AFM (Veeco Instruments Inc., Santa Barbara, CA), the sample was immobilized by mechanical trapping on a 0.8-μm polycarbonate membrane (Millipore) (Oh *et al.*, 2013) and imaged in contact mode with a DNP-10 cantilever (Bruker, Vienna, Austria) with a nominal spring constant of 0.06 N m⁻¹ and a nominal tip diameter of 20 nm. Scan line speed was 1 Hz.

**Biochemical methods**

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of OMVs and crude *T. forsythia* cell extract was performed on 12% slab gels in a Mini Protean electrophoresis apparatus (Bio-Rad, Vienna, Austria) (Laemmli, 1970). Proteins were stained with colloidal Coomassie Brilliant Blue R-250 and glycans were visualized with periodic acid Schiff reagent. Western-blotting of (glyco)proteins to a polyvinylidene difluoride membrane using anti-TfsA and anti-TfsB specific polyclonal antiserum was performed as described elsewhere (Steiner *et al.*, 2007). Relative quantification of S-layer proteins using OMV preparations equaling 5 μg, 0.5 μg and 0.05 μg of total protein was performed directly from the Western blots using an Odyssey scanner (LI-COR, Bad Homburg, Germany) and integration of the intensity signal obtained from the S-layer bands at 700 nm using the application software 3.0.

The protein content of the samples was determined with the 2-D Quant kit (GE Healthcare, Vienna, Austria). To determine the LPS content of the OMV preparation used in the cell culture experiments, the amount of 3-deoxy-α-manno-oct-2-ululosonic acid (Kdo) was calculated (Warren, 1959).

**Mass spectrometry of OMVs**

The OMV preparations from three independent biological replicates were separated in 10-μl aliquots, each, by SDS–PAGE. Three zones were excised per lane and proteins were S-alkylated with iodoacetamide and subsequently subjected to in-gel digestion with modified trypsin (Promega, Mannheim, Germany). The peptide mixture was analyzed in the positive-ion DDA mode (switching to MSMS mode for eluting peaks) using a Dionex Ultimate 3000 system (Thermo Scientific, Vienna, Austria) directly linked to a quadrupole time-of-flight mass spectrometer (MS) (Bruker maxis 4G ETD; Bruker) equipped with the captive spray source and nano-booster set-up. MS scans were recorded over a range of 150–2200 Da and
the eight highest peaks were selected for fragmentation. Instrument calibration was performed using the ESI calibration mixture (Agilent, Santa Clara, CA). For separation of the peptides, a Thermo Acclaim PepMap300 RSLC C18 separation column (2-μm particle size; 150 × 0.075 mm) was used with a Thermo Acclaim PepMap μ-pre-column. A gradient from 5% to 32% of solvent B (0.1% formic acid in acetonitrile) in solvent A (0.1% formic acid in HQ-water) within 60 min was applied at a flow rate of 0.3 μl min⁻¹, followed by a 10-min gradient from 32% B to 70% B to promote elution of large peptides.

Bioinformatic protein identification

The analysis files from MS were converted (using Data Analysis, Bruker) to XML files suitable for performing an MS/MS ion search with ProteINScape (Mascot). The tandem mass spectrometry data were assigned to the protein sequences of T. forsythia ATCC 43037 as annotated by the NCBI Prokaryotic Genome Annotation Pipeline. The Mascot search results of each gel lane were merged. Positive hits were accepted with at least two peptides per protein and a Mascot score of at least 30. In Table 1, only proteins that could be identified in all three replicate samples are listed. The total Mascot score was averaged across all samples and gel lanes.

To find orthologs of the ATCC 43037 strain within the widely used 92A2 (ATCC BAA-2717) genome, pBLAST was used to search all ‘Tanf’ ATCC 43037 protein sequences against both the ‘TF’ Oralgen (http://www.oralgen.org) and the ‘BFO’ NCBI (http://www.ncbi.nlm.nih.gov) annotated proteomes. In addition to an E-value cut-off of 1 × 10⁻⁵, the parameters ‘best_hit_score_edge 0.1’ and ‘best_hit_overhang 0.1’ were applied to select for only the best hits (BLAST Command Line Applications User Manual http://www.ncbi.nlm.nih.gov/books/NBK21097).

Function prediction was performed using PANNZER (bitscore threshold 50, identity threshold 50%, query and target coverage 0.6) (Koskinen et al., 2015). ScanPROSITE (http://prosite.expasy.org/scanprosite) was used to identify potential glycosylation sites by searching the T. forsythia proteome for the phylumwide glycosylation motif D(S,T) (A,L,V,I,M,T) (Coyne et al., 2013).

Proteins detected in the T. forsythia OMVs were classified according to their subcellular localization, which was established using the bacterial localization prediction software CELLO (http://meme-suite.org/tools/glam2scan) (Yu et al., 2004).

The prediction of CTD proteins was conducted by running a BLASTP analysis of known T. forsythia CTD proteins in strain 92A2 (Veith et al., 2009b) against the new genome of strain ATCC 43037. Furthermore, using previously described CTD sequences, a GLAM2 motif was created and subsequently used in GLAM2Scan (http://meme-suite.org/tools/glam2scan) to confirm positive hits.

Cell lines

The U937 human monocytic cell line was purchased from ATCC and primary human periodontal ligament fibroblasts (hPdLFs) isolated from 16-year old male donors were purchased from Lonza (Basel, Switzerland). Both cell lines were cultured in RPMI-1640
medium (Invitrogen, Vienna, Austria) and Dulbecco’s modified Eagle’s medium (Invitrogen), respectively, supplemented with 10% of fetal calf serum (FCS), 100 U ml\(^{-1}\) penicillin, and 100 μg ml\(^{-1}\) streptomycin at 37°C in a humidified atmosphere containing 5% CO\(_2\). For differentiation into adherent macrophages, U937 cells were seeded (10\(^6\) cells ml\(^{-1}\)) and treated with phorbol 12-myristate 13-acetate (Sigma) as described elsewhere (Sekot et al., 2011).

**Stimulation of macrophages and human gingival fibroblasts with OMVs**

Adherent U937 macrophages were seeded in a 24-well plate at a density of 2 × 10\(^5\) cells per well containing 0.5 ml of RPMI-1640 medium, and hPdLFs were seeded at a density of 5 × 10\(^4\) cells per well containing 0.5 ml of Dulbecco’s modified Eagle’s medium; either medium was additionally supplemented with 10% FCS. After 24 h, the media were replaced with low-serum media (1% FCS) containing different amounts of OMVs (corresponding to 0.1 μg, 1.0 μg and 10 μg protein per ml).

In a parallel set of experiments, human soluble CD14 (sCD14, 0.25 μg ml\(^{-1}\); Sigma, St Louis, USA) was added during stimulation. Cells stimulated with viable wild-type *T. forsythia* (10\(^7\) cells ml\(^{-1}\)) (Bodet et al., 2006) were used as a positive control. Each experimental group included three wells. After stimulation of U937 for 3 h and of hPdLFs for 24 h, the cellular mRNA expression levels of TNF-α and IL-8 in macrophages, and of IL-6, IL-8 and MCP-1 in hPdLFs, as well as the content of the corresponding proteins in the conditioned medium were determined. Experiments were repeated at least three times.

**Viability test of mammalian cells**

U937 macrophages and hPdLFs were treated with OMVs as described under ‘Stimulation of macrophages and human gingival fibroblasts with OMVs’ above. Additionally, PBS was used as a control. Each experimental group included eight wells. After stimulation, 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye (5 mg ml\(^{-1}\) in PBS) was added and culture plates were incubated at 37°C for 4 h. Subsequently, the medium was discarded, 100 μl of dimethylsulfoxide was added, and the OD\(_{550}\) was measured on a Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA).

**Quantitative polymerase chain reaction**

The mRNA expression levels of TNF-α, IL-6, IL-8, and MCP-1 were determined by quantitative polymerase chain reaction (qPCR), with glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) and β-actin serving as internal references. Isolation of mRNA and transcription into cDNA was performed using the Taq-Man Gene Expression Cells-to-CT kit (Ambion/Applied Biosystems, Foster City, CA), which provides good accuracy and superior sensitivity of gene expression analysis (Van Peer et al., 2012). The qPCR was performed on an ABI StepOnePlus device (Life Technologies, Carlsbad, CA) in paired reactions using the TaqMan gene expression assays with the following ID numbers (Life Technologies): TNF-α, Hs00174103_m1; IL-6, Hs00985639_m1; IL-8, Hs00174103_m1; MCP-1, Hs00234140_m1; GAPDH, Hs99999905_m1; β-actin, Hs99999903_m1. The qPCR were performed in triplicate as described previously (Sekot et al., 2011). The point at which the PCR product was first detected above a fixed threshold (cycle threshold, C\(_T\)), was
determined for each sample. Changes in the expression of target genes were calculated using the $2^{-\Delta\Delta C_{\text{t}}}$ method, using an untreated sample as control (Sekot et al., 2011).

**Determination of cytokines**
The levels of TNF-α, IL-6, IL-8, and MCP-1 in the conditioned media were determined by enzyme-linked immunosorbent assay using Ready-SET-Go kits (eBioscience, San Diego, CA). For measurement of TNF-α and IL-8 production by U937 macrophages, samples were diluted at a ratio of 1: 10 and 1: 100, respectively; for measurements of IL-6 and MCP-1 production by hPdLFs samples were used undiluted and for measurement of IL-8 production samples were diluted at a the ratio of 1: 5. The detection limit for all cytokines was 2 pg ml$^{-1}$.

**Statistical analysis**
The statistical difference between groups was determined by analysis of variance, while paired comparisons were performed using Tukey’s post-hoc test. All statistical analyses were performed using the statistics program SPSS 20.0. Data are expressed as mean ± SD. Differences were considered to be statistically significant at $P < 0.05$.

**RESULTS AND DISCUSSION**

**Microscopy of *T. forsythia* OMVs**
The TEM of ultrathin-sectioned *T. forsythia* cells grown in serum-free brain-heart infusion broth with a mean generation time of 16 h$^{-1}$ revealed that OMV formation occurred by budding off from the OM, with the S-layer completely covering the nascent vesicles (Fig. 1A). The S-layer of OMVs exhibits a thickness of 22 nm, which conforms to the value measured from whole *T. forsythia* cells (Sekot et al., 2012). The presence of an intact S-layer was supported by analysis of a negatively stained preparation of isolated OMVs, on which the square S-layer lattice symmetry typical of *T. forsythia* cells was visible (Fig. 1B). The analyses of OMVs from intact OMV-secreting cells as well as purified preparations revealed an almost uniform diameter of ~100 nm (Fig. 1C,D). These findings are supported by non-invasive AFM in vitro imaging of OMV-secreting *T. forsythia* cells, where cells neither received any chemical treatment nor were subjected to a drying process, which may trigger undesirable biological side effects (Oh et al., 2013). There, OMVs could be clearly visualized in the immediate surroundings as well as lying on top of the imaged *T. forsythia* cell (Fig. 1E).

**Biochemical analyses of *T. forsythia* OMVs**
The *T. forsythia* OMV preparation used in the course of this study revealed an overall protein concentration of 2 μg ml$^{-1}$, based on the 2-D Quant kit, and a Kdo content of 16 ng ml$^{-1}$.

The presence of an S-layer as the outermost layer of the *T. forsythia* OMVs (compare with Fig. 1) was further supported by the detection of both high molecular-mass S-layer glycoproteins, TfsA-GP and TfsB-GP, on a Coomassie Brilliant Blue-stained SDS–PAGE
gel (not shown) and on a Western immunoblot using anti-TfsA and anti-TfsB antibody, respectively (Fig. 2).

Considering the demonstrated involvement of the S-layer in the immune response to *T. forsythia* (Sekot et al., 2011), it was important to quantify the S-layer content of the OMVs in relation to intact *T. forsythia* cells to assess the inflammatory response triggered by the OMVs (see below). Densitometric quantification of the two S-layer glycoproteins TfsA-GP and TfsB-GP from the Western immunoblot revealed that the amount of S-layer in the OMV preparation with a total protein content of 5 μg equaled that present on $10^7$ *T. forsythia* cells.

**Proteomics**

To obtain a detailed composition of *T. forsythia* ATCC 43037 OMVs, we performed a liquid chromatography-MS/MS analysis of three independent biological replicates upon separation by SDS–PAGE, resulting in the high-confidence identification of 208, 202 and 192 proteins, respectively. Protein hits that were exclusively detected in one (31 hits) or two replicates (23 hits) were disregarded. In all, 175 proteins were found in all three replicates and were selected for further bioinformatic analyses (Table 1). These represent 76% of all identified proteins, implying good analytical and biological reproducibility (see Supplementary material, Table S1).

We decided to use a 1D gel shotgun proteomics approach to avoid potential pitfalls of frequently used techniques based on 2D-gel electrophoresis, where specific non-ionic or zwitterionic detergents are required in isoelectric focusing in which highly hydrophobic membrane proteins are relatively insoluble (Braun et al., 2007).

**The strain issue**—Due to an error in strain attribution, all previous studies on *T. forsythia* ATCC 43037 reported in the literature are based on the genome of a different strain, *T. forsythia* 92A2 (which has only recently been deposited at ATCC under the strain number BAA-2717). To complicate matters, most references to *T. forsythia* genes and proteins in the literature use the initial annotation that was performed by the Los Alamos National Laboratory (http://www.oralgen.org), which is different from the annotation that can be found at NCBI (http://www.ncbi.nlm.nih.gov). All our data refer to the recently sequenced strain ATCC 43037 and the new annotation for the genes (Tanf) is used.

As this is the first study to use the correct genome sequence, we searched for orthologs of the identified proteins in both the NCBI (BFO) and Oralgen (TF) annotations and included all respective locus tags in Table S1. Although some parts of the genomes of the two strains (ATCC 43037 vs. 92A2) show considerable differences (Friedrich et al., 2015), all but two proteins present in the OMVs have putative orthologs in strain 92A2.

**Functional annotation**—A combination of the inference of protein function via homology and a prediction using PANNZER (Koskinen et al., 2015) suggested functions for 97 OMV proteins, whereas the remaining 78 hits were classified as hypothetical or were only assigned general domain descriptions. When categorized according to COG functional classes (http://www.ncbi.nlm.nih.gov/COG), most proteins found in OMVs are involved in inorganic ion transport and metabolism (25 hits), post-translational modification, protein
turnover and chaperone function (12 hits), carbohydrate transport and metabolism (7), cell motility, secretion and intracellular trafficking (7), cell envelope and OM biogenesis (6) and amino acid transport and metabolism (4). The majority of proteins, a total of 106 (60%), could not be placed into any COG class (Table S1).

**Prediction of subcellular protein localization**—The prediction of subcellular localization using the software CELLO places 61 proteins (35%) in the OM, 53 (30%) in the periplasm and classifies 22 proteins (13%) as extracellular proteins. When compared with the subcellular distribution within the entire *T. forsythia* proteome (Fig. 3; see Supplementary material, Table S1), OMVs are highly enriched in OM and periplasmic proteins whereas, as expected, components predicted to be associated with the inner membrane are completely excluded. Thirty-nine proteins (22%) are predicted to be localized to the cytoplasm – however, of these, 12 have been previously identified as components of the *T. forsythia* OM proteome (Veith et al., 2009b), indicating the limitations of *in silico* subcellular localization prediction.

As several proteomic analyses have shown that OMVs can contain cytoplasmic proteins, either through targeted incorporation or accidental inclusion, a number of proteins predicted to originate from the cytoplasm are to be expected (Lee et al., 2008). Our initial OMV purifications contained two particularly abundant cytoplasmic proteins, elongation factor EF-Tu and molecular chaperone GroEL, both of which have been frequently found in other OMV proteome studies (Ohara et al., 2006). Even though it is often suggested to use density gradient centrifugation or gel filtration to enhance purity, we found that harvesting the OMVs from a younger culture (here, a batch culture at OD$_{600}$ ~1.0) and including additional washing steps eliminated the presence of these two proteins alongside any ribosomal components.

**Glycoproteins**—Based on our previous finding that *T. forsythia* modifies many of its proteins with the unique S-layer glycan (Posch et al., 2011) and that *T. forsythia* manipulates the cytokine responses of macrophages and monocytes through its surface glycosylation (Settem et al., 2013) as well as its biofilm formation capacity (Honma et al., 2007), it was interesting to see whether the OMVs would contain glycosylated cargo apart from the S-layer glycoproteins. To identify potential glycoproteins, we therefore searched the protein hits for the phylum-wide glycosylation motif D (S,T)(A,L,V,I,M,T) (Coyne et al., 2013). A total of 80 proteins (46%) contain at least one occurrence of the sequon, with the S-layer protein TfsB (Tanf_03375) and the hypothetical protein Tanf_00535 featuring the most (16 predicted sites). Although the number of identified OMV proteins comprise only 7% of the annotated proteome, they include 27% of all proteins that have a higher number of glycosylation motifs (i.e. six or more).

To the best of our knowledge, previous studies have identified a total of 16 glycoproteins in *T. forsythia*, either through the proteomic analysis of carbohydrate-stained bands on 2D-gels (Veith et al., 2009b) or the detection of the S-layer oligosaccharide from proteins subjected to reductive β-elimination (Posch et al., 2011). Using whole cell extracts of *T. forsythia*, we identified four more proteins carrying the S-layer glycan in the course of this study (data not shown). Out of these 20 glycoproteins, we could detect 18 in the OMVs (Table 2).
glycoproteins found in the OMV fraction include, besides the two S-layer proteins TfsA and TfsB (11 and 16 predicted glycosylation sites), and the major surface antigen BspA (one predicted glycosylation site), a possible hemagglutinin/hemolysin (Tanf_06020; six predicted glycosylation sites) and a putative zinc-protease (Tanf_03780; two predicted glycosylation sites) that has also been found to be preferentially packed into the OMVs of *Bacteroides fragilis* (Elhenawy et al., 2014) and *P. gingivalis* (Veith et al., 2014). Also present, and presumably in the lumen of the OMVs, is a tetratricopeptide-domain-containing protein (Tanf_07305) that shows homology to PG1385 (TprA) from *P. gingivalis*, which is upregulated in mouse subcutaneous infection (Yoshimura et al., 2008). Of the 18 OMV glycoproteins, 10 are hypothetical. These include Tanf_00990, Tanf_11855, Tanf_00065, Tanf_02425 and Tanf_08965, all of which have been described as particularly abundant in the OM (Veith et al., 2009b).

**CTD proteins**—In *P. gingivalis*, a close phylogenetic relative of *T. forsythia* affiliated to the *Bacteroidales* order of bacteria (Conrads et al., 2005; Coyne et al., 2013) and member of the ‘red-complex’ consortium of periodontal pathogens (Socransky et al., 1998), the newly identified T9SS is dependent on a CTD in certain proteins for their successful secretion and surface attachment (Glew et al., 2012). These CTD proteins, along with other virulence factors, were later found to be considerably enriched in the OMVs (Veith et al., 2014).

Two recent studies provided the first evidence of a T9SS in *T. forsythia* and simultaneously confirmed the importance of CTD signals for the secretion of the two S-layer proteins (Narita et al., 2014; Tomek et al., 2014). To determine to which degree CTD proteins were present within the OMV preparation, we compared all putative CTD proteins from strain 92A2 (Veith et al., 2013) to their respective orthologs in strain ATCC 43037 using BLASTP and also directly searched for CTD-specific motifs with GLAM2Scan. Of the 26 putative CTD proteins that we could identify in the entire *T. forsythia* ATCC 43037 proteome (see Supplementary material, Table S1), 14 were detected in the OMVs. Of these, seven were already reported to be part of the OM proteome (Veith et al., 2009b), while the other seven, mostly hypothetical proteins, could be exclusive to the OMV preparation. Four proteins, Tanf_00065, Tanf_00990, Tanf_08965 and Tanf_11855, show high sequence similarity to the N-terminal region of previously reported CTD proteins (Narita et al., 2014) but lack the C-terminal OM translocation signal itself.

As expected, several components of the OM-bound T9SS could also be found in our analysis of the OMV fraction, including orthologs of PorQ (TF1959), PorT (TF0188), the putative C-terminal signal peptidase PorU (TF0955) and LptO (TF2852). In *P. gingivalis*, the integral OM protein LptO has been shown to be essential for the coordinated secretion of CTD proteins (Chen et al., 2011) and was found to be among the most abundant vesicle cargo components in OMVs (Veith et al., 2014).

Among the protein hits with the highest Mascot score were the CTD-containing S-layer glycoproteins TfsA-GP and TfsB-GP, the surface antigen BspA and the hypothetical high-molecular-weight glycoprotein Tanf_02425. BspA is a member of the leucine-rich repeat and bacterial immunoglobulin-like protein families; it is associated with the cell surface of *T. forsythia* and functions as an important modulator of host innate immune responses.
through activation of TLR2 in cooperation with TLR1 (Onishi et al., 2008). Also, the virulence-related possible hemagglutinin/hemolysin (Tanf_06020) is a CTD-protein.

**TonB-associated OM proteins**—As expected, components of the OM were the most numerous among all OMV protein hits. Ninety-nine (57%) of all identified OMV proteins were already detected in the published OM proteome of *T. forsythia* 92A2 (Veith et al., 2009b).

Among them are 40 proteins that are classified as being associated with TonB-dependent transport, 30 of which show similarity to gene products of the *susB* operon (including SusC, SusD and SusE) of *Bacteroides thetaiotaomicron*. These OM proteins form transporter complexes with TonB-dependent receptors that are likely to be involved in the import of carbohydrates and other nutrients (Lee et al., 2008). One particular SusD family protein, Tanf_11260 (TF1416 in strain 92A2) has been identified as an *in vivo* antigen during human infection (Galka et al., 2008).

Within this group are also orthologs (Tanf_13710 and Tanf_13705) of a recently described TonB-dependent OM sialic acid transport system in strain 92A2 consisting of the neuraminate OM permease NanO (TF0033) and the extracellular neuraminate uptake protein NanU (TF0034) (Banerji et al., 2008). Another component of *T. forsythia*’s sialic acid utilization locus, the sialidase NanH, could also be found in OMVs (Tanf_13700). This enzyme allows *T. forsythia* to scavenge sialic acid from human glycoconjugates, with the cleaved sialic acid acting as an important nutrient for bacterial growth as well as being key to the initial formation and maturation of biofilms (Roy et al., 2011).

We also identified a homolog of HmuY, a TonB-dependent receptor-associated OM lipoprotein that was found to be highly enriched in the vesicle membrane of *P. gingivalis* OMVs (Veith et al., 2014).

Interestingly, while all four aforementioned proteins could be readily detected in all three replicates of our analysis, none of them has been previously described as part of the OM proteome.

**Hydrolitic enzymes**—A previous investigation of two different *Bacteroides* species revealed that acidic glycosidases and proteases were preferentially and selectively packed into their OMVs (Elhenawy et al., 2014). While our analysis did not allow us to determine whether controlled enrichment of components took place, we detected a high number of proteins annotated to possess hydrolitic functions, many of which were not found previously in the OM proteome and could therefore constitute virulent OMV cargo. Apart from the sialidase NanH that was mentioned earlier, the OMV fraction contained another sialidase, SiaHI (Horstman & Kuehn, 2000) and the β-N-acetylglucosaminidase HexA (Hughes et al., 2003), both of which are suspected to be involved in biofilm formation (Yoshimura et al., 2008). The failure to detect the recently described α-L-fucosidase of *T. forsythia* in the bacterium’s OMVs (Megson et al., 2015), might be explained by its biological role in the catabolism of short oligosaccharides in the periplasm, thereby only indirectly contributing to the virulence of *T. forsythia*.
In addition to orthologs of five periplasmic peptidases that have been described as constituents of the OM proteome (Veith et al., 2009b), we identified seven additional peptidases. Four of them, the dipeptidase Tanf_00250, the zinc protease Tanf_03780, the putative peptidase Tanf_11165 and the aminopeptidase Tanf_13550 exhibit high homology with hydrolases that have been found exclusively in the OMVs of both B. fragilis (Elhenawy et al., 2014) and P. gingivalis (Veith et al., 2014). Another periplasmic protease, Tanf_11420, is annotated as HtrA, a secreted virulence factor in Helicobacter pylori. HtrA has been shown to facilitate the invasion of intact epithelium by cleaving the cell-adhesion protein E-cadherin, thereby allowing persistent H. pylori colonization and pathogenesis (Hoy et al., 2010). A recent study identified a group of putative secretory proteases that share a nearly identical C-terminal domain ending with a Lys-Leu-Ile-Lys-Lys motif and that are consequently referred to as KLIKK proteases (Ksiazek et al., 2015b). Of these, the metalloprotease forsilysin (Tanf_06225) and the serine protease miropsin-2 (Tanf_06530) were found in the OMV proteome. Both enzymes have been shown to possess the ability to degrade elastin, an important component of connective tissue, and could therefore play an active role in periodontal lesions. In contrast to our finding of miropsin-2 and forsilysin in the OMV fraction, these enzymes were detected as soluble forms in the particle-free culture supernatant of T. forsythia cells (Narita et al., 2014).

Tanf_05640 is annotated as a xylanase, an enzyme that was also found in the OMVs of the cellulolytic bacterium Fibrobacter succinogenes (formerly in the genus Bacteroides), where it is believed to contribute to polymer digestion and nutrient acquisition (Forsberg et al., 1981). It is conceivable that the xylanase ortholog in T. forsythia is one of several hydrolytic enzymes involved in the scavenging of nutrients.

The lysophospholipase Tanf_10855 belongs to a family of lipolytic proteins that share a distinct GDSL motif in their active site. In the pathogen Legionella pneumophilia, a related enzyme, lysophospholipase A, was found in the OMV fraction and could be involved in the mediation of the fusion processes between OMVs and their target membranes (Galka et al., 2008). Apart from their role as virulence factors in many pathogenic bacteria, phospholipases can also create cleavage products that are used in the bacteria’s lipid metabolism (Banerji et al., 2008).

Other hydrolytic enzymes that were detected in the OMVs include a β-galactosidase (Tanf_02010), an endonuclease (Tanf_02020), a phosphohydrolase (Tanf_13640), a putative metallopeptidase (Tanf_08225) similar to thermolysin, and an alkaline phosphatase (Tanf_00095). The periplasmic enzyme alkaline phosphatases has been reported in the OMVs of various species (Horstman & Kuehn, 2000; Bomberger et al., 2009; Evans et al., 2012) and is considered a major virulence factor in Pseudomonas aeruginosa.

Considering that OMVs of different species have been reported to contain a wide range of hydrolytic enzymes, it is not surprising to find adaptations to the highly proteolytic environment of subgingival plaque that T. forsythia inhabits. In our analyses, we detected an ortholog of miropin (Tanf_07655), a bacterial serpin that has been recently reported to inhibit a wide range of proteases and that may function as an important virulence factor by protecting the bacterium from the destructive activity of neutrophil serine proteases (Chen et
al., 2005). While the subcellular localization prediction in our study classifies miropin as a cytoplasmic protein, Ksiazek et al. suggest that it is most likely exposed to the extracellular environment on the bacterial cell surface with a fraction also present in the periplasm (Ksiazek et al., 2015c).

Other proteins—Among the other integral OM proteins that could be detected in the OMV preparation is an ortholog to the major OmpA-like protein (Tanf_10935) which has been shown to be antigenic but most likely not exposed on the surface (Veith et al., 2009b). Furthermore, we could find a protein that exhibits high sequence similarity to lipoprotein PG3 of P. gingivalis (Tanf_00475) and that has been identified as an antigen specifically expressed in patients with periodontal disease (Yoo et al., 2007).

Interestingly, an ortholog of the most abundant soluble protein in the lumen of P. gingivalis OMVs, signal protein PDZ, could also be found in our OMV analysis (Tanf_10630).

Superoxide dismutase activity predicted to be encoded by Tanf_04530 has been shown to be important in the obligate anaerobe P. gingivalis for protection against aerobic exposure (Ohara et al., 2006). The Fe/Mn-containing superoxide dismutase SodF in T. forsythia has been shown to be under the regulation of a redox-sensor which exerts an influence over both antioxidant responses and synergistic biofilm formation with Fusobacterium nucleatum (Honma et al., 2009).

Unclassified proteins—Despite using an automated protein function prediction method that was ranked highly in a recent, largescale evaluation (Radivojac et al., 2013), more than half of all proteins that were detected in the OMV preparation could not be assigned any function. Current protein databases are faced with exponentially growing sequencing information but a lack of experimentally verified protein annotations. Therefore, the automated annotation of proteins of uncertain function remains a challenging process (Mills et al., 2015).

Nevertheless, two unclassified proteins that were found in the OMVs are worth mentioning: the two tetratricopeptide repeat (TPR) domain proteins Tanf_07305 and Tanf_09615 are orthologous to proteins identified as soluble cargo in the vesicle lumen of P. gingivalis OMVs (Veith et al., 2014). One of them, the TPR-domain protein TprA (PG1385), has recently been shown to be involved in the pathogen’s virulence. The tprA gene was upregulated in P. gingivalis wild-type cells placed in a mouse subcutaneous chamber and a tprA knock-out mutant was clearly less virulent (Kondo et al., 2010).

Inflammatory response in U-937 macrophages and hPdLFs upon stimulation with OMVs

Before assaying the inflammatory response and to exclude a toxic effect of the purified OMVs on the cell lines used in this study, the viability of U937 macrophages and hPdLFs upon challenge with OMVs at concentrations of 0.03–10 μg ml⁻¹ was tested (data not shown). In U937 macrophages, OMVs induced a dose-dependent decrease in macrophage viability. This effect was significant for all tested OMV concentrations. The viability of hPdLFs was significantly increased by OMVs at concentrations ranging from 1 to 10 μg ml⁻¹, whereas lower OMV concentrations had no significant effect on hPdLF viability.
In U-937 macrophages, in the absence of sCD14, OMVs induced a concentration-dependent increase in mRNA and protein levels of TNF-α and IL-8. The presence of sCD14 in conditioned media significantly increased TNF-α production levels in response to 0.1–1 μg ml⁻¹ OMVs and IL-8 production in response to 0.1 μg ml⁻¹ OMVs. The response of U937 to 1–10 μg ml⁻¹ of OMVs was significantly higher than that to whole T. forsythia (10⁷ cells ml⁻¹) (Fig. 4).

Similarly, in hPdLFs, in the absence of sCD14, OMVs induced a concentration-dependent increase of IL-6, IL-8 and MCP-1 expression, on both gene and protein levels. The presence of sCD14 significantly increased both gene and protein levels of all proinflammatory mediators in response to 0.1–1 μg ml⁻¹ OMV as well as protein production in response to 10 mg ml⁻¹ OMV. The IL-8 and MCP-1 response to 10 μg ml⁻¹ OMV was significantly higher than that to whole T. forsythia (10⁷ cells ml⁻¹) (Fig. 5).

In the present study, we focused on the measurements of the expression of those proinflammatory mediators that are thought to play an important role in the progression of peritonitis. TNF-α may directly stimulate bone resorption in vitro and in vivo (Mundy, 1993); or stimulate production of prostaglandin E₂ (Nakao et al., 2002; Rausch-Fan et al., 2005), which is a potent stimulator of bone resorption (Offenbacher et al., 1993). Interleukin-6 is a pro-inflammatory cytokine, which plays a key role in acute inflammation phase and promotes bone resorption (Ishimi et al., 1990; Fonseca et al., 2009). Interleukin-8 and MCP-1 are chemoattractants that induce migration of neutrophils and monocytes, respectively, to the inflammation site and promote the development of acute inflammation (Bagnioli et al., 1994; Silva et al., 2007).

Our data showed that the response of macrophages and hPdLFs to an OMV challenge strongly depends on the presence of CD14 in the conditioned media. CD14 is a glycosylphosphatidylinositol-anchored protein, but it also exists in soluble form (Kim et al., 2005). CD14 together with the LPS-binding protein is thought to capture the LPS molecule and transfer it to TLR4 (Park et al., 2009). Hence, dependency of OMV response on the presence of sCD14 suggests the involvement of LPS-signaling in this inflammatory response. The effect of sCD14 was more pronounced in hPdLF than in macrophages. This observation was not surprising, considering that macrophages are hematopoietic cells, which usually express high levels of membrane-bound CD14 (Jersmann, 2005), whereas hPdLFs usually express low levels of membrane-bound CD14 (Hatakeyama et al., 2003). It is known that soluble CD14 is present in gingival crevicular fluid and its levels might change in periodontal disease (Jin & Darveau, 2001).

CONCLUSIONS

It is established knowledge that the periodontal pathogen T. forsythia has developed sophisticated strategies for host evasion and virulence, including molecular mimicry of host tissues to avoid immune detection, modulation of the host immune response, and entry into host cells to escape immune surveillance (Amano et al., 2014). Key to these mechanisms is, among others, the decoration of T. forsythia’s cell surface with a unique glycan linked to its
S-layer proteins whose synthesis relies on the general protein O-glycosylation pathway of the bacterium that also targets other Tannerella proteins (Posch et al., 2011, 2012).

In this study, we characterized T. forsythia OMVs as a new addition to the bacterium’s virulence repertoire and simultaneously investigated, for the first time, OMV biogenesis of an S-layer covered bacterium. It was shown that OMVs with a narrow size distribution of ~100 nm in diameter are formed by budding off from the OM while retaining an intact, glycosylated S-layer (compare with Fig. 1). Hence, it is conceivable that the S-layer serves its important functions in mediation of cell adhesion and invasion (Sakakibara et al., 2007) as well as in immune evasion (Sekot et al., 2011; Settem et al., 2013, 2014) also in conjunction with OMVs as a prerequisite for the delivery of virulent cargo to host cells.

Shotgun proteomics using liquid chromatography matrix assisted laser desorption/ionization time-of-flight/time-of-flight identified 175 proteins, many of which have an attributed virulence function, are (predicted) glycoproteins or both (Table 1; see Supplementary material, Table S1). Considering that OMVs selectively sort their cargo it is likely that these glycoproteins would serve important roles in OMV functionality, given the importance for virulence that has already been attributed to the T. forsythia O-glycan (Settem et al., 2013, 2014).

Despite the clear enrichment of OM and periplasmic proteins in the T. forsythia OMVs alongside glycoproteins, the preparations were predicted to contain a few cytoplasmic proteins, with the numbers varying depending on the used algorithm for subcellular localization prediction. Although we consider a contamination of the OMV preparation from other subcellular compartments as unlikely, we cannot exclude at the current stage of investigation that the preparation contained small amounts of inner OM vesicles. These were recently described as a new type of membrane vesicles of Gram-negative pathogens such as Neisseria gonorrhoeae, Pseudomonas aeruginosa and Acinetobacter baumanii, occurring in minor amounts (0.2–1.5% of total vesicles produced) and typically also containing cytoplasmic components (Pérez-Cruz et al., 2015). On the other hand, although the number of cytoplasmic proteins found in an OMV preparation is often used as a measure of its purity, the presence of such proteins is not necessarily an indication of contamination with cellular material (Ohara et al., 2006).

The virulent character of T. forsythia OMVs was supported by the measurement of the release of the proinflammatory mediators TNF-α and IL-8 in macrophages and IL-6, IL-8 and MCP-1 in hPdLFs. Cytokine release was dependent on the concentration of OMVs used for stimulation and, in all cases, was significantly higher than the response to a challenge with whole T. forsythia cells. To elucidate the mechanisms underlying these observed responses, future studies will focus on the virulence potential of particular glycosylated OMV cargo.

In conclusion, our study represents the first characterization of T. forsythia OMVs, their proteomic composition and immunogenic potential. Our results suggest that T. forsythia OMVs could play a role in the shuttling of virulence factors to affect the development and progression of periodontal disease.
Acknowledgements

The authors thank Andrea Scheberl (Department of NanoBiotechnology) and Nguyen Phuong Quynh (Medical University of Vienna) for excellent technical assistance. Financial support came from the Austrian Science Fund FWF, project P24317-B22 (to C.S.), International Team of Implantology, project 781_2011 (to O.A.), and the PhD program ‘BioToP–Biomolecular Technology of Proteins’ (Fund, FWF project W1224).

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Figure 1.
Transmission electron microscope images (A–D) and atomic force microscopy image (E) of *Tannerella forsythia* ATCC 43037 wild-type cells and outer membrane vesicles (OMVs). (A) Ultrathin-sectioned preparation of a cell showing the biogenesis of an OMV (white triangle) from the outer membrane and coverage of the nascent OMV with the S-layer. (B) Negatively-stained preparation of isolated *T. forsythia* OMVs showing the square S-layer lattice (inset) and (C) the size distribution of the OMVs. (D) Negatively stained and (E) untreated, physically trapped *T. forsythia* cell showing nascent OMVs in the immediate surroundings of the cells. S, S-layer; OM, outer membrane; CM, cytoplasmic membrane.
Figure 2.
Western blot analysis for detection of the S-layer glycoproteins TfsA-GP and TfsB-GP in the outer membrane vesicle preparation (OMV) and, for comparison, on whole *Tannerella forsythia* ATCC 43037 cells (Tf cells), using anti-TfsA (A) and anti-TfsB (B) antibodies. The S-layer proteins were subsequently quantified using the LI-COR Odyssey scanner. MW, molecular weight marker PageRuler Plus Prestained Protein Ladder (Thermo Scientific, Vienna, Austria).
Figure 3.
Comparison of subcellular localization prediction of the whole proteome and the outer membrane vesicle (OMV) fractions of *Tannerella forsythia* ATCC 43037.
Figure 4.
Effect of *Tannerella forsythia* ATCC 43037 outer membrane vesicles (OMVs) on the production of tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8) in U937 macrophages. Expression levels of TNF-α (A) and IL-8 (B) in U-937 macrophages upon 3 h of stimulation with OMVs (0.1–10 μg ml⁻¹) in the presence or absence of sCD14 (0.25 μg ml⁻¹). Cells stimulated with *T. forsythia* (10⁷ cells ml⁻¹) were used as positive control. Left panels show gene-expression levels measured by quantitative polymerase chain reaction. The y-axis represents the n-fold expression levels of the target gene in relation to non-stimulated cells. Right panels show the protein content in the conditioned media after stimulation measured by enzyme-linked immunosorbent assay. Data are presented as mean ± SEM. *significantly higher vs. control; ‡significantly higher vs. positive control (*T. forsythia*, 10⁷ cells ml⁻¹); #significantly higher vs. corresponding group without sCD14.
Figure 5.
Effect of *Tannerella forsythia* ATCC 43037 outer membrane vesicles (OMVs) on the production of interleukin-6 (IL-6), IL-8 and monocyte chemotactic protein 1 (MCP-1) by hPdLCs. Expression levels of TNF-α (A) and IL-8 (B) in human periodontal ligament fibroblasts (hPdLFs) upon 24 h of stimulation with OMVs (0.1–10 μg ml⁻¹) in the presence or absence of sCD14 (0.25 μg ml⁻¹). Cells stimulated with *T. forsythia* (10⁷ cells ml⁻¹) were used as positive control. Left panels show gene-expression levels measured by quantitative polymerase chain reaction. The y-axis represents the n-fold expression levels of target gene in relation to non-stimulated cells. Right panels show protein content in the conditioned
media after stimulation measured by enzyme-linked immunosorbent assay. Data are presented as mean ± SEM. *significantly higher vs. control; †significantly higher vs. positive control (T. forsythia, $10^7$ cells ml$^{-1}$); ‡significantly higher vs. corresponding group without sCD14.
### Table 1

**Identification and localization of outer membrane vesicle (OMV) proteins**

| Locus tag  | Description                                      | MW (kDa) | Glyco-sites | Predicted location | Ortholog in strain 92A2 | Mascot score | Previously reported in OM proteome |
|------------|--------------------------------------------------|----------|-------------|--------------------|--------------------------|--------------|------------------------------------|
| Tanf_03375 | surface layer protein TisB                       | 150.4    | 16* P       | TF2663             | 2735                     | +            |                                    |
| Tanf_04820 | surface antigen BspA                             | 123.3    | 1* P        | TF1843             | 2330                     | +            |                                    |
| Tanf_03370 | surface layer protein TisA                       | 133.1    | 11* OM      | TF2861             | 2052                     | +            |                                    |
| Tanf_06020 | possible hemagglutinin/hemolysin                 | 131.2    | 6* E        | TF2116             | 1381                     | +            |                                    |
| Tanf_03675 | alkyl hydroperoxide reductase                    | 39.1     | 1           | TF2730             | 730                      |              |                                    |
| Tanf_10675 | hypothetical protein                             | 41.9     | 4           | TF1273             | 659                      |              |                                    |
| Tanf_05655 | hypothetical protein                             | 58.2     | 0           | TF1552             | 512                      |              |                                    |
| Tanf_12435 | immune reactive antigen PG93                    | 84.8     | 0           | TF1952             | 393                      |              |                                    |
| Tanf_05510 | hypothetical protein                             | 41.1     | 3 OM        | TF1514             | 358                      |              |                                    |
| Tanf_02330 | hypothetical protein                             | 200.0    | 13 E        | TF3202             | 253                      | +            |                                    |
| Tanf_10605 | hypothetical protein                             | 34.0     | 0 C         | TF2339             | 232+                     |              |                                    |
| Tanf_03310 | bacterial group 2 Ig-like protein                | 41.0     | 9 P         | TF2645             | 174                      |              |                                    |
| Tanf_12195 | hypothetical protein                             | 38.8     | 0 OM        | TF1896             | 103                      |              |                                    |
| Tanf_00535 | hypothetical protein                             | 212.7    | 16 E        | TF3163             | 88+                      |              |                                    |
| Tanf_04930 | peptidase, S41 family                            | 54.1     | 3 OM        | TF3024             | 842+                     |              |                                    |
| Tanf_11420 | periplasmic serine protease HprA                 | 54.2     | 0 OM        | TF1450             | 762                      |              |                                    |
| Tanf_00250 | peptidase PepD, family C69                       | 61.8     | 1 P         | TF0298             | 513                      |              |                                    |
| Tanf_04010 | probable secreted glycosyl hydrolase            | 32.6     | 3* P        | TF2804             | 439+                     |              |                                    |
| Tanf_00095 | alkaline phosphatase                             | 62.7     | 1 P         | TF0338             | 437                      |              |                                    |
| Tanf_10930 | peptidase, S41 family                            | 107.5    | 2* C        | TF2753             | 374                      |              |                                    |
| Tanf_02020 | endonuclease                                     | 31.5     | 0 C         | TF1471             | 307                      |              |                                    |
| Tanf_01221 | peptidase family M49                            | 78.4     | 1 C         | TF2531             | 291+                     |              |                                    |
| Tanf_04515 | β-N-acetylgalactosaminidase HexA                 | 86.7     | 2 C         | TF2925             | 290+                     |              |                                    |
| Tanf_09025 | peptidase, S41 family                            | 48.8     | 2 OM        | TF1243             | 282                      |              |                                    |
| Tanf_08225 | metalloendopeptidase PepO                       | 77.3     | 3 P         | TF1033             | 267+                     |              |                                    |
| Tanf_10855 | lyso phospholipase                              | 28.8     | 2 P         | TF3133             | 262                      |              |                                    |
| Tanf_07615 | putative alkaline protease AprF                  | 52.1     | 0 C         | TF0773             | 261+                     |              |                                    |
| Tanf_05646 | xylanase                                         | 32.3     | 0 C         | TF1549             | 236                      |              |                                    |
| Tanf_13640 | lcc family phosphohydrolase                     | 36.2     | 0 C         | TF0948             | 221                      |              |                                    |
| Tanf_11165 | peptidase S10                                    | 81.4     | 2 C         | TF1398             | 183                      |              |                                    |
| Tanf_07795 | glycosyl hydrolase                              | 48.5     | 1 C         | TF0813             | 166+                     |              |                                    |
| Tanf_13700 | sialidase (neuraminidase) NanH                   | 59.7     | 2 OM        | TF0035             | 132                      |              |                                    |
| Tanf_08515 | peptidase, S41 family                            | 39.1     | 2 C         | TF1755             | 122+                     |              |                                    |
| Tanf_13550 | aminopeptidase C1-like family                   | 45.1     | 0 E         | TF0078             | 109                      |              |                                    |
| Tanf_06530 | miropsin-2                                       | 80.2     | 0 OM        | TF0357             | 104                      |              |                                    |
| Tanf_02565 | peptidase S41                                   | 121.8    | 0 P         | TF0959             | 97+                      |              |                                    |
| Tanf_06225 | forsi lysin                                     | 91.3     | 2 E         | TF2162             | 94                       |              |                                    |
| Tanf_02010 | β-galactosidase                                  | 128.4    | 1 E         | TF1468             | 78                       |              |                                    |

*C-terminal-domain-containing proteins*

*Hydrolitic enzymes*
### Locus tag | Description | MW (kDa) | Glyco-sites | Predicted location | Ortholog in strain 92A2 | Mascot score | Previously reported in OM proteome
--- | --- | --- | --- | --- | --- | --- | ---
Tanf_02130 | exo-α-sialidase SiaHI | 51.0 | 1 | P | TF2207 | 41 | +

**Type IX secretion system proteins**

Tanf_04220 | integral OM protein LptO | 43.6 | 0 | OM | TF2852 | 1025 | +
Tanf_02580 | putative C-terminal signal peptidase | 127.6 | 3 | OM | TF0955 | 750 | +
Tanf_12465 | PoreQ protein | 37.2 | 0 | OM | TF1959 | 184 | +
Tanf_10520 | PoreT protein | 27.1 | 0 | OM | TF0188 | 102 | +

**TonB-associated outer membrane proteins**

Tanf_09365 | neuraminidate OM permease NanO | 121.5 | 0 | OM | TF0033 | 3000 | +
Tanf_08335 | OM protein Omp121 | 113.7 | 0 | OM | TF1057 | 2063 | +
Tanf_13730 | neuraminidate uptake protein NanU | 59.5 | 3 | OM | TF0034 | 1880 | +
Tanf_09365 | SusC/RagA family TonB-linked OM protein | 117.9 | 0 | OM | TF2193 | 1328 | +
Tanf_09370 | SusC/RagA family TonB-linked OM protein | 76.2 | 0 | OM | TF2192 | 1313 | +
Tanf_09520 | TonB-dependent receptor HmuY | 24.5 | 0 | P | TF3077 | 1088 | +
Tanf_10785 | TonB-linked OM protein | 118.0 | 0 | OM | TF3104 | 1009 | +
Tanf_02485 | SusC/RagA family TonB-linked OM protein | 117.7 | 1 | OM | TF0976 | 939 | +
Tanf_08320 | OM protein Omp121 | 117.4 | 0 | OM | TF1053 | 890 | +
Tanf_13410 | SusC/RagA family TonB-linked OM protein | 110.3 | 0 | OM | TF0313 | 866 | +
Tanf_13440 | SusC/RagA family TonB-linked OM protein | 58.7 | 0 | OM | TF0992 | 858 | +
Tanf_13475 | OM protein SusC | 112.3 | 0 | OM | TF0093 | 835 | +
Tanf_03665 | SusC/RagA family TonB-linked OM protein | 133.1 | 1 | OM | TF2728 | 807 | +
Tanf_06285 | SusC/RagA family TonB-linked OM protein | 110.9 | 0 | OM | TF2348 | 759 | +
Tanf_07090 | SusC/RagA family TonB-linked OM protein | 45.3 | 0 | C | TF3103 | 678 | +
Tanf_00185 | SusC/RagA family TonB-linked OM protein | 64.5 | 0 | P | TF0312 | 595 | +
Tanf_00330 | SusC/RagA family TonB-linked OM protein | 61.6 | 0 | P | TF0277 | 520 | +
Tanf_06290 | SusC/RagA family TonB-linked OM protein | 61.6 | 0 | P | TF3249 | 511 | +
Tanf_00940 | OM protein Omp121 | 116.3 | 0 | OM | TF2417 | 468 | +
Tanf_09960 | SusC/RagA family TonB-linked OM protein | 118.6 | 0 | OM | TF2412 | 431 | +
Tanf_03670 | SusC/RagA family TonB-linked OM protein | 54.9 | 0 | C | TF2729 | 419 | +
Tanf_02480 | SusC/RagA family TonB-linked OM protein | 63.5 | 0 | C | TF0977 | 408 | +
Tanf_12670 | SusC/RagA family TonB-linked OM protein | 129.2 | 0 | OM | TF0588 | 381 | +
Tanf_13485 | OM protein SusE | 41.8 | 1* | E | TF0091 | 376 | +
Tanf_00335 | SusC/RagA family TonB-linked OM protein | 111.7 | 0 | OM | TF0275 | 357 | +
Tanf_05475 | SusC/RagA family TonB-linked OM protein | 60.5 | 0 | OM | TF1505 | 324 | +
Tanf_11255 | TonB-dependent receptor FtrG, OM protein | 121.4 | 0 | OM | TF1415 | 280 | +
Tanf_01380 | SusC/RagA family TonB-linked OM protein | 114.0 | 0 | OM | TF2032 | 276 | +
Tanf_07645 | SusC/RagA family TonB-linked OM protein | 54.2 | 0 | P | TF0779 | 256 | +
Tanf_00170 | SusC/RagA family TonB-linked OM protein | 87.3 | 1 | OM | TF0318 | 252 | +
Tanf_07640 | SusC/RagA family TonB-linked OM protein | 114.1 | 0 | OM | TF0778 | 212 | +

* Mol Oral Microbiol. Author manuscript; available in PMC 2015 December 01.
| Locus tag     | Description                                           | MW (kDa) | Glyco-sites | Predicted location | Ortholog in strain 92A2 | Mascot score | Previously reported in OM proteome |
|--------------|-------------------------------------------------------|----------|-------------|--------------------|-------------------------|--------------|-----------------------------------|
| Tanf_06060   | probable TonB-linked OM protein                       | 68.4     | 2           | OM                 | TF2124                  | 208          | +                                 |
| Tanf_13670   | TonB-dependent siderophore receptor                  | 88.2     | 4           | OM                 | TF0041                  | 199          | +                                 |
| Tanf_00240   | TonB-dependent receptor                               | 97.3     | 2           | OM                 | TF0301                  | 191          | +                                 |
| Tanf_01385   | SusD/RagB family OM lipoprotein                      | 62.2     | 1           | E                  | TF2031                  | 176          | +                                 |
| Tanf_12665   | SusD family protein                                  | 58.1     | 0           | P                  | TF0587                  | 167          | +                                 |
| Tanf_03660   | SusD family protein                                  | 65.3     | 0           | OM                 | TF2727                  | 95           | +                                 |
| Tanf_11260   | SusD family protein                                  | 65.3     | 0           | P                  | TF1416                  | 79           | +                                 |
| Tanf_07035   | SusD family protein                                  | 67.3     | 1           | OM                 | TF0483                  | 60           | +                                 |
| Tanf_07030   | SusC/RagA family TonB-linked OM protein              | 119.6    | 0           | OM                 | TF0482                  | 46           | +                                 |
| Tanf_09620   | Outer membrane proteins                              |          |             |                    |                         |              |                                   |
| Tanf_01080   | OmpA-like OM protein, OmpA family                    | 121.1    | 0           | OM                 | TF2096                  | 321          | +                                 |
| Tanf_09805   | OM protein assembly complex                           | 102.6    | 3           | OM                 | TF2450                  | 227          | +                                 |
| Tanf_10935   | OM protein 40, OmpA-like                             | 44.4     | 1           | P                  | TF1331                  | 180          | +                                 |
| Tanf_01635   | copper resistance lipoprotein NilpE                  | 15.9     | 0           | C                  | TF1158                  | 126          | +                                 |
| Tanf_09795   | cationic OM protein Omph                             | 19.0     | 1           | P                  | TF2452                  | 117          |                                   |
| Tanf_00475   | possible immunogenic lipoprotein                     | 23.9     | 0           | P                  | TF0015                  | 103          | +                                 |
| Tanf_06695   | OM assembly lipoprotein YifO                         | 31.9     | 0           | OM                 | TF0403                  | 71           |                                   |
| Tanf_11435   | Sugar phosphate isomerase/epimerase                  | 33.8     | 0           | C                  | TF1454                  | 967          |                                   |
| Tanf_04005   | oxidoreductase domain protein                        | 54.8     | 1           | P                  | TF2096                  | 963          | +                                 |
| Tanf_10630   | PDZ domain signal protein                            | 54.9     | 1           | OM                 | TF1262                  | 518          |                                   |
| Tanf_07655   | protease inhibitor miropin                           | 45.7     | 0           | C                  | TF0781                  | 490          |                                   |
| Tanf_13170   | putative peptidyl-prolyl isomerase                   | 61.7     | 4*          | C                  | TF2506                  | 426          |                                   |
| Tanf_07420   | sporulation and cell division repeat protein         | 17.7     | 0           | P                  | TF1733                  | 406          | +                                 |
| Tanf_09265   | glutathione peroxidase                               | 22.6     | 0           | P                  | TF2342                  | 357          |                                   |
| Tanf_04530   | superoxide dismutase SodF                            | 22.1     | 0           | C                  | TF2927                  | 338          |                                   |
| Tanf_02155   | hypothetical protein                                 | 21.6     | 0           | P                  | TF2214                  | 297          | +                                 |
| Tanf_05520   | alkyl hydroperoxide reductase                        | 22.5     | 0           | P                  | TF1518                  | 284          |                                   |
| Tanf_13665   | anaerobic cobalt chelatase ChsK                      | 35.3     | 0           | C                  | TF0042                  | 245          |                                   |
| Tanf_00220   | peptidyl-prolyl cis-trans isomerase SlyD              | 26.5     | 2           | P                  | TF0305                  | 210          | +                                 |
| Tanf_13180   | peptidyl-prolyl cis-trans isomerase SurA             | 52.5     | 3           | C                  | TF2504                  | 203          |                                   |
| Tanf_13630   | flavoxxdin                                          | 21.1     | 0           | P                  | TF0054                  | 176          |                                   |
| Tanf_08600   | aldose 1-epimerase GalM                              | 41.3     | 3           | E                  | TF1798                  | 148          |                                   |
| Tanf_00150   | possible YngK protein                                | 60.4     | 1           | C                  | TF0322                  | 89           | +                                 |
| Tanf_07430   | oxidoreductase domain protein                        | 47.2     | 0           | P                  | TF1735                  | 86           |                                   |
| Tanf_00210   | hydroxypyruvate isomerase                            | 33.0     | 0           | C                  | TF0307                  | 74           |                                   |
| Tanf_03930   | hypothetical protein                                 | 23.2     | 0           | P                  | none                    | 1315         |                                   |
| Tanf_12375   | tetratricopeptide repeat protein                     | 62.0     | 0           | P                  | TF1940                  | 1285         | +                                 |

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| Locus tag | Description | MW (kDa) | Glyco-sites | Predicted location | Ortholog in strain 92A2 | Mascot score | Previously reported in OM proteome |
|-----------|-------------|----------|-------------|-------------------|-------------------------|--------------|----------------------------------|
| Tanf_08330 | tetra-| 71.2 | 2* P | TF1056 | 1220 + |
| Tanf_09615 | tripeptide repeats | 51.2 | 1 P | TF0704 | 1072 |
| Tanf_04855 | OM protein β-barrel domain protein | 22.6 | 0 OM | TF3007 | 951 + |
| Tanf_11390 | hypothetical, putative hemin receptor | 60.4 | 0 OM | TF1444 | 879 + |
| Tanf_13105 | Cna protein B-type domain protein | 48.8 | 0 E | TF0683 | 864 + |
| Tanf_07305 | tetra- | 46.5 | 1* P | TF0548 | 777 |
| Tanf_10775 | putative internalin A | 39.3 | 0 P | TF1294 | 773 |
| Tanf_02630 | putative surface protein | 40.4 | 2 E | TF0945 | 704 + |
| Tanf_13015 | hypothetical protein | 29.5 | 1 P | TF0661 | 694 + |
| Tanf_08325 | hypothetical protein | 32.6 | 2 P | TF1055 | 618 + |
| Tanf_08965 | hypothetical protein | 171.4 | 11* E | TF1259 | 453 + |
| Tanf_10985 | possible lipoprotein | 54.5 | 6* OM | TF1342 | 452 + |
| Tanf_10190 | hypothetical protein | 27.8 | 2 C | TF2537 | 429 |
| Tanf_03150 | hypothetical protein | 44.6 | 0 P | TF2606 | 407 + |
| Tanf_06055 | tetra- | 114.5 | 2 OM | TF2123 | 394 + |
| Tanf_08315 | hypothetical protein | 67.5 | 2 E | TF1052 | 391 + |
| Tanf_10595 | hypothetical protein | 54.0 | 2 C | TF0358 | 390 |
| Tanf_03025 | hypothetical protein | 17.7 | 0 C | TF1714 | 390 |
| Tanf_06535 | hypothetical protein | 23.0 | 0 E | TF0358 | 376 |
| Tanf_09820 | putative lipoprotein | 51.2 | 5 OM | TF2447 | 366 + |
| Tanf_09445 | hypothetical protein | 16.0 | 0 P | TF0365 | 362 + |
| Tanf_06545 | hypothetical protein | 16.2 | 0 P | TF0365 | 350 + |
| Tanf_07845 | hypothetical protein | 21.7 | 0 P | TF0827 | 324 |
| Tanf_09945 | hypothetical protein | 58.1 | 0 P | TF2416 | 321 + |
| Tanf_13280 | hypothetical protein | 63.0 | 0 OM | TF2485 | 310 |
| Tanf_12860 | hypothetical protein | 54.8 | 0 OM | TF0627 | 299 |
| Tanf_05505 | hypothetical protein | 54.1 | 0 OM | none | 296 |
| Tanf_00990 | hypothetical protein | 61.5 | 2* E | TF2592 | 293 + |
| Tanf_02915 | hypothetical protein | 56.1 | 2 C | TF1689 | 273 |
| Tanf_12975 | hypothetical protein | 79.0 | 5 OM | TF0652 | 259 + |
| Tanf_05585 | hypothetical protein | 18.2 | 0 P | TF1534 | 256 + |
| Tanf_12970 | hypothetical protein | 36.2 | 0 OM | TF0651 | 255 |
| Tanf_11375 | hypothetical protein | 23.5 | 0 OM | TF1441 | 244 + |
| Tanf_01450 | hypothetical protein | 17.7 | 0 P | TF2016 | 228 + |
| Tanf_05560 | hypothetical protein | 13.5 | 0 P | TF1527 | 210 |
| Tanf_01050 | hypothetical protein | 20.1 | 0 C | TF0860 | 205 |
| Tanf_00065 | hypothetical protein | 65.2 | 2* E | TF1741 | 200 + |
| Tanf_12120 | hypothetical protein | 26.0 | 0 OM | TF1857 | 199 |
| Tanf_03695 | hypothetical protein | 23.0 | 0 OM | TF2734 | 178 + |
| Tanf_12265 | hypothetical protein | 25.1 | 0 C | TF1911 | 176 |
| Tanf_12880 | bacterial group 2 Ig-like protein | 27.4 | 0 P | TF0631 | 175 |
| Tanf_09950 | hypothetical protein | 25.3 | 1 OM | TF2415 | 168 + |
| Tanf_03830 | hypothetical protein | 29.2 | 2 C | TF2764 | 161 |
| Tanf_07425 | hypothetical protein | 126.6 | 0 P | TF1734 | 153 |

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| Locus tag    | Description                          | MW (kDa) | Glyco-sites | Predicted location | Ortholog in strain 92A2 | Mascot score | Previously reported in OM proteome |
|-------------|--------------------------------------|----------|-------------|--------------------|--------------------------|--------------|-----------------------------------|
| Tanf_00445  | hypothetical protein                  | 15.6     | 0           | C                  | TF0342                   | 148          |                                   |
| Tanf_00810  | hypothetical protein                  | 31.2     | 0           | E                  | TF1754                   | 142          |                                   |
| Tanf_07010  | hypothetical protein                  | 39.8     | 0           | C                  | TF0479                   | 139          |                                   |
| Tanf_06555  | hypothetical protein                  | 13.3     | 0           | C                  | TF0368                   | 129 +        |                                   |
| Tanf_11855  | hypothetical protein                  | 67.4     | 3*          | E                  | TF2592                   | 124 +        |                                   |
| Tanf_13010  | hypothetical protein                  | 49.4     | 1           | OM                 | TF0660                   | 123          |                                   |
| Tanf_10145  | WD40-like protein                    | 54.4     | 3           | C                  | TF2369                   | 116          |                                   |
| Tanf_00660  | hypothetical protein                  | 44.7     | 3           | C                  | TF3133                   | 114          |                                   |
| Tanf_07095  | hypothetical protein                  | 52.2     | 0           | P                  | TF0495                   | 111          |                                   |
| Tanf_10060  | hypothetical protein                  | 55.8     | 1           | P                  | TF2390                   | 107          |                                   |
| Tanf_08090  | hypothetical protein                  | 23.5     | 0           | P                  | TF1003                   | 92           |                                   |
| Tanf_03090  | hypothetical protein                  | 33.1     | 6           | C                  | TF2594                   | 88           |                                   |
| Tanf_00865  | hypothetical protein                  | 32.6     | 2           | P                  | TF3086 <sup>5</sup>      | 87           |                                   |
| Tanf_05230  | hypothetical protein                  | 21.8     | 0           | C                  | TF0137                   | 83           |                                   |
| Tanf_07265  | bacterial group 2 Ig-like protein     | 38.4     | 1           | E                  | TF0540                   | 82           |                                   |
| Tanf_09955  | hypothetical protein                  | 26.1     | 2*          | P                  | TF2414                   | 81 +         |                                   |
| Tanf_03095  | hypothetical protein                  | 59.1     | 1           | OM                 | TF2595                   | 77 +         |                                   |
| Tanf_11080  | hypothetical protein                  | 39.7     | 2           | C                  | TF1392                   | 67           |                                   |

1 Number of occurrences of the phylum-wide glycosylation motif. Asterisks denote proteins that have been reported to be glycosylated.

2 CELLO prediction of subcellular localization: Periplasmic (P), Outer membrane (OM), Extracellular (E), Cytoplasmic (C).

3 Total Mascot score averaged across the replicate samples.

4 Veith et al. (2009b).

5 More than one possible ortholog found, only best hit shown.
### Table 2

Glycoproteins identified in *Tannerella forsythia* ATCC 43037 outer membrane vesicles

| ATCC locus tag | Description                                      | MW (kDa) | Glyco sites | CTD present | References                         |
|----------------|--------------------------------------------------|----------|-------------|-------------|------------------------------------|
| Tanf_03375     | S-layer protein TfsB                              | 150.4    | 16          | +           | Veith et al. (2009b); Posch et al. (2011) |
| Tanf_04820     | surface antigen BspA                              | 123.3    | 1           | +           | Veith et al. (2009b)               |
| Tanf_03370     | S-layer protein TfsA                              | 133.1    | 11          | +           | Veith et al. (2009b); Posch et al. (2011) |
| Tanf_02425     | hypothetical protein                              | 159.9    | 13          |             | Veith et al. (2009b); Posch et al. (2011) |
| Tanf_06020     | possible hemagglutinin/hemolysin                  | 131.2    | 6           | +           | Veith et al. (2009b)               |
| Tanf_08330     | tettraticopeptide repeat protein                  | 71.2     | 2           |             | Veith et al. (2009b); Posch et al. (2011) |
| Tanf_07305     | tettraticopeptide repeat protein                  | 46.5     | 1           |             | our experiments                    |
| Tanf_08965     | hypothetical protein                              | 171.4    | 11          |             | Veith et al. (2009b); Posch et al. (2011) |
| Tanf_10985     | possible lipoprotein                              | 54.5     | 6           |             | Veith et al. (2009b)               |
| Tanf_04010     | probable secreted glycosylhydrolase               | 32.6     | 3           |             | Veith et al. (2009b)               |
| Tanf_13170     | putative peptidyl-prolyl isomerase                | 61.7     | 4           |             | our experiments                    |
| Tanf_13485     | OM protein SusE                                   | 41.8     | 1           |             | Posch et al. (2011)                |
| Tanf_03780     | peptidase, M16 family                             | 107.5    | 2           |             | our experiments                    |
| Tanf_00990     | hypothetical protein                              | 61.5     | 2           |             | Veith et al. (2009b)               |
| Tanf_00065     | hypothetical protein                              | 65.2     | 2           |             | Veith et al. (2009b)               |
| Tanf_11855     | hypothetical protein                              | 67.4     | 3           |             | Veith et al. (2009b)               |
| Tanf_09795     | cationic OM protein OmpH                          | 19.0     | 1           |             | our experiments                    |
| Tanf_09955     | hypothetical protein                              | 26.1     | 2           |             | Veith et al. (2009b)               |