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Protein secretion systems in *Fusobacterium nucleatum*: Genomic identification of Type 4 piliation and complete Type V pathways brings new insight into mechanisms of pathogenesis

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Abstract

Recent genomic analyses of the two sequenced strains *F. nucleatum* subsp. *nucleatum* ATCC 25586 and *F. nucleatum* subsp. *vincentii* ATCC 49256 suggested that the major protein secretion systems were absent. However, such a paucity of protein secretion systems is incongruous with *F. nucleatum* pathogenesis. Moreover, the presence of one or more such systems has been described for every other Gram-negative organism sequenced to date. In this investigation, the question of protein secretion in *F. nucleatum* was revisited. In the current study, the absence in *F. nucleatum* of a twin-arginine translocation system (TC #2.A.64.), a Type III secretion system (TC #3.A.6.), a Type IV secretion system (TC #3.A.7.) and a chaperone/usher pathway (TC #1.B.11.) was confirmed. However, contrary to previous findings, our investigations indicated that a Type I protein secretion system was also absent from *F. nucleatum.* In contrast, members of the holin family (TC #1.E) and the machinery required for a Type 4 piliation/fimbriation system (TC #3.A.15.2.) were identified using a variety of bioinformatic tools. Furthermore, a complete range of proteins resembling members of the Type V secretion pathway, i.e., the Type Va (autotransporter; TC #1.B.12.), Type Vb (two-partner secretion system; TC #1.B.20.) and Type Vc (YadA-like trimeric autotransporter; TC #1.B.42.), was found. This work provides new insight into the protein secretion and virulence mechanisms of *F. nucleatum*.
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Keywords: Protein secretion; Type V secretion pathway; Autotransporter; Two-partner secretion system; YadA-like autotransporter; Fusobacterium; Type 4 pili

1. Introduction

Fusobacterium nucleatum is an obligate anaerobic Gram-negative spindle-shaped or fusiform bacillus, belonging to the phylum *Fusobacteria*, class *Fusobacteria*, order *Fusobacteriales*, family *Fusobacteriaceae* and is the type species for the genus *Fusobacterium* [1,2]. *Fusobacterium nucleatum* is currently divided into three subspecies: *F. nucleatum* subsp. *nucleatum, F. nucleatum* subsp. *polymorphum* and *F. nucleatum* subsp. *vincentii* [2]. The genome of the type strain *F. nucleatum* subsp. *nucleatum* ATCC 25586 has been recently published [3] while the draft genome of *F. nucleatum* subsp. *vincentii* ATCC 49256 is currently being assembled [4].

F. nucleatum is the most common Gram-negative bacterium isolated from periodontal sites and is a key micro-organism in oral microbiology since it is a common human dental plaque isolate, playing a crucial role in plaque development, and is also frequently associated with the periodontal diseases gingivitis and periodontitis, as well as tonsillitis or invasive human infections of the head and neck, chest, lung, liver and abdomen [5–8]. Gingivitis is

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associated with the formation of a bacterial biofilm, i.e., dental plaque, at the gum–tooth interface and subsequent inflammation of the gingival tissue. Gingivitis is the forerunner to destructive periodontal disease which is characterised by loss of gingival tissue, resulting in detachment of the gingiva from the tooth, alveolar bone resorption and eventual tooth loss. *F. nucleatum* is known (i) to adhere to tooth surfaces and gingival cells [9,10], (ii) to act as a co-aggregation bridge micro-organisms allowing establishment of other pathogens, and thus a heterogenous biofilm in the periodontal pocket [10], (iii) to invade host tissue cells [11] and (iv) to modulate the host immune response [12,13]. However, the molecular mechanisms by which *F. nucleatum* achieves these pathogenic steps, and thus how it contributes to gingivitis and periodontal disease, are poorly understood [10].

Like all micro-organisms, the capacity of *F. nucleatum* to infect mammalian hosts depends on the ability to secrete virulence factors which are either displayed on the bacterial cell surface, secreted in the extracellular milieu or even injected directly into the host cell. In Gram-negative bacteria, six major protein secretion systems are currently recognised, i.e., the Type I, II, III, IV, V and the chaperone/usher pathway [14–23]. A recent report suggested that aside from a Type I secretion pathway, the other major protein secretion systems were missing from *F. nucleatum* [4]. Such a paucity of protein secretion systems seems puzzling and incongruous when considering the pathogenesis of *F. nucleatum* and cannot adequately explain the exceptional adhesive ability of this micro-organism. Thus, reinvestigation of the *F. nucleatum* genomes for protein secretion systems was justified.

In contrast to the previous studies, and as presented here, comprehensive analyses of the genome sequence data of *F. nucleatum* subsp. *nucleatum* ATCC 25586 and *F. nucleatum* subsp. *vincentii* ATCC 49256 revealed the presence of a Type 4 pilus (Tfp) locus and a complete range of genes encoding Type V secretion systems, which include (i) the Type Va, including the classical autotransporter proteins (AT-1; TC #1.B.12.), (ii) the Type Vb, representing the two-partner secretion pathway (TPS; TC #1.B.20.), and (iii) the Type Vc, i.e., the YadA-like trimeric autotransporters (AT-2; TC #1.B.42.) [23–26]. The Tfp have been associated with adhesion and biofilm formation in a variety of Gram-negative organisms, while the Type V secretion pathway represents the largest protein secretion family in Gram-negative bacteria and appears to be a premium source of virulence factors [21,23,27–30]. Furthermore, unlike the previous studies, we were unable to identify a Type I protein secretion system. In addition, translocation of proteins through the inner membrane relies only on the Sec (Secretion) apparatus since components of the twin-arginine translocation (Tat) system are absent. This work provides a novel insight into the protein secretion systems and pathogenesis of *F. nucleatum*.

2. Materials and methods

2.1. Bioinformatic analysis of protein secretion systems

Prior to bioinformatic analyses, the complete protein sequence database of *F. nucleatum* subsp. *nucleatum* ATCC 25586 and *F. nucleatum* subsp. *vincentii* ATCC 49256 were downloaded from National Center for Biotechnology Information (NCBI) ftp site (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/Fusobacterium_nucleatum/) and NCBI unfinished genome sequences database (http://www.ncbi.nlm.nih.gov/genomes/static/eub_u.html), respectively and are referred to according to their GenBank index number (GI) and gene locus tag.

All predicted ORFs of *F. nucleatum* subsp. *nucleatum* ATCC 25586 and *F. nucleatum* subsp. *vincentii* ATCC 49256 were analysed using the Transporter Classification Database (TC-DB; http://www.biology.ucsd.edu/~msaier/transport/) as previously described [31]. For investigations of the Type I secretion pathway, the analysis was completed by combining results from TransportDB [32] a relational database describing the predicted cytoplasmic membrane transport proteins in bacteria (http://66.93.129.133/transporter/wb/index2.html) and ABCdb (http://www.lcb.cnrs-mrs.fr/~quentin/) a public database devoted to *Archaea* and *Bacteria* ABC transporters.

The presence of signal peptides was determined using SignalP (http://www.cbs.dtu.dk/services/SignalP/) [33], PSORT (http://www.psort.ims.u-tokyo.ac.jp/form.html) [34], and LipoP (http://www.cbs.dtu.dk/services/LipoP/) [35]. Subcellular localisation of the proteins was predicted using PSORTb (http://www.psort.org/psortb/) [36].

BLAST and PSI-BLAST [37,38] were performed from ViruloGenome (http://www.vge.ac.uk/). The percentage of identity between two protein sequences was determined using BLAST 2 sequences using the matrix BLOSUM62 and default parameters [39] from the National Center for Biotechnology Information web site (NCBI; http://www.ncbi.nlm.nih.gov/). Six frame translation of DNA sequences were performed from Baylor College of Medecine Search Launcher (http://searchlauncher.bcm.tmc.edu/) [40]. DNA protein coding region were found using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Sequence alignments were performed using ClustalW [41] under BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). For phylogenetic analysis, sequence alignments were subjected to distance matrix and neighbour joining methods using ClustalX (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) [42]. The resulting phylogenetic trees were visualised with TreeExplorer developed by Dr. K. Tamura (http://evolgen.biol.metro-u.ac.jp/TE/TE_man.html). Bootstrap values were calculated for 1000 replicates and given in percentage.

Functional motifs were searched using Pfam (http://www.sanger.ac.uk/SoftwarePfam/) [43], InterProScan (http://www.ebi.ac.uk/InterProScan/) [44], CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) [45], ProDom (http://

www.prodes.toulouse.inra.fr/prodom/2002.1/htmlhome.php) [46] and ScanProsite (http://www.ca.expasy.org/tools/scanprosite/) [47].

The probable secondary structure of proteins was inspected combining several programs namely BetaWrap for β-helix prediction (http://betawrap.lcs.mit.edu/betawrap.html) [48,49], PRED-TMBB and AMPHI for β-barrel prediction (http://www.bioinformatics.biol.uoa.gr/PRED-TMBB/ and http://www.vge.ac.uk/-amphi/, respectively) [50,51], COILS for coiled-coil prediction (http://www.ch.embnet.org/software/COILS_form.html) [52], AMPHI and PSIPRED for α-helix and β-strands prediction (http://bioinf.cs.ucl.ac.uk/psipred/) [53,54].

Supplementary material is available at the following address: http://xbase.bham.ac.uk/Fusobacteria/.

3. Results

3.1. The protein translocation systems in F. nucleatum

Table 1 summarises the protein secretion systems found in the two subspecies of *F. nucleatum* examined. While the original annotation of *F. nucleatum* genomes was done with ERGO™, the bioinformatic suite of Integrated Genomics, and was essentially based on sequence similarities using FASTA algorithm, motif/pattern databases Pfam, Prosite, ProDom and COGs as well as Integrated Genomics proprietary tools [3,4], the present study employed manual annotation to identify the correct start and termination codons for each ORF and involved several other bioinformatic tools including ones especially designed for the analysis of protein secretion-related systems as described in the Materials and methods. Reinvestigation of the protein secretion systems in *F. nucleatum* following TC-DB searches confirmed the apparent absence of (i) a twin-arginine translocation (Tat) system (TC #2.A.64), (ii) a Type III secretion system (TC #3.A.6), i.e., flagella export apparatus, injectisome or Hrp pilus export apparatus, (iii) a Type IV secretion system (TC #3.A.7) and (iv) a chaperone/usher pathway (TC #1.B.11). It is worth mentioning that the proteins annotated as TatD in *F. nucleatum*, i.e., FN1343 and FNV1467, are cytoplasmic deoxyribonucleases and have no obligate involvement in protein export by the Tat system [55].

In *F. nucleatum*, as with most bacteria characterised to date, the Sec translocon appears to be the main pathway permitting protein translocation into the periplasm [56]. In Gram-negative bacteria, SecB and SRP are considered as two distinct protein-targeting pathways converging on the Sec translocon [57]. Interestingly, while homologues of the signal recognition particle (SRP) pathway, i.e., Ffh and FtsY, were identified, SecB homologues could not be found in *F. nucleatum*. Importantly, based on 16S rRNA sequence analysis, the genus *Fusobacterium* is related to the Gram-positive *Corynebacterium–Nocardia–Mycobacterium* complex [58]. In all Gram-positive bacteria, SecB is absent [59], and in *B. subtilis*, the SecB dual function of preventing folding and targeting the protein to the Sec translocon is attributed to CsaA (BSU19040; 16078964); nevertheless, no homologue of CsaA could be identified in *F. nucleatum*. In *Escherichia coli*, alternative chaperones, namely DnaJ, DnaK, GroES and GroEL which are all present in *F. nucleatum* (i.e., FN0118, FN0116, FN0676 and FN0675, respectively), have been shown to contribute to protein translocation through the Sec apparatus [60,61]; however, the recognition mechanism involved in targeting of the exported protein to the Sec translocon remains elusive. Given the evidence mentioned above, it is unlikely that in the absence of a SecB homologue that *F. nucleatum* relies exclusively on SRP for inner membrane protein translocation, rather identification of a chaperone fulfilling a role similar to the *E. coli* SecB or *B. subtilis* CsaA is awaited.

Besides the Sec translocon, two other families of cytoplasmic membrane proteins involved in inner membrane protein translocation could be identified in *Fusobacterium*, i.e., members of the holin family (TC #1.E) and members of the family of large conductance mechanosensitive ion channel (MscL; TC #1.A.22) (Table 1). Because of the small number of proteins translocated by these pathways, holins and MscL are rather considered as anecdotal protein translocation systems [31]. Members of the LrgA/CidA holin family (TC #1.E.14) could be identified in both *F. nucleatum* subsp. *nucleatum* ATCC 25586 and *F. nucleatum* subsp. *vincentii* ATCC 49256. Holins are small membrane proteins whose function is mainly associated with permeabilisation of the cytoplasmic membrane with concomitant protein export [62,63]. In *Staphylococcus aureus*, *lrg* and *cid* operons are involved in regulation of murein hydrolase activity in a process apparented to programmed cell death, where the murein hydrolase exporters CidA and LrgA would function as holin and antiholin and LrgB and CidB as murein hydrolase export regulators and/or murein hydrolases [63,64]. Aside from protecting bacterial cells from osmotic downshock, members of the MscL family of large conductance mechanosensitive ion channel are also known to permit the release of small proteins such as thioredoxin, EF-Tu or DnaK during osmotic downshift [65–67]. An MscL homologue (FN0766) could be identified in *F. nucleatum* subsp. *nucleatum* ATCC 25586 (Table 1). While a second LrgB/CidB homologue and MscL homologue could not be identified in *F. nucleatum* subsp. *vincentii*, this genome is incomplete and finishing and assembly of the genome sequence may reveal homologues at a later date.

The ABC superfamily contains both uptake and efflux transport systems for a large variety of substrates, i.e., glucides, lipids, proteins, amino acids, peptides, ions. As previously reported and confirmed by ABCdb, TransportDB and TC-DB analyses, several ABC transporters are present in *F. nucleatum* [3,4]. However, an ABC transporter (TC #3.A.1) alone cannot permit protein secretion into the

Table 1
The protein components of the potential protein secretion pathways in *Fusobacterium*

Systems	Components	TC[a]	Predicted Cellular location[b]	Locus name (GI)[c]	
				F. nucleatum subsp. *nucleatum* ATCC 25586	*F. nucleatum* subsp. *vincentii* ATCC 49256
1. The Sec pathway		#3.A.5			
Transmembrane component	SecY homologue	#3.A.5	IM	FN1624 (19704945)	FNV0270 (34764209)[d]
	SecE homologue	#3.A.5	IM	FN2042 (19705333)	FNV1855 (34762877)
	SecG homologue	#3.A.5	IM	FN0538 (19703873)	FNV1379 (34763259)
					FNV0321 (34764156)[d]
ATPase	SecA homologue		P	FN1718 (19705039)	FNV0218 (34764869)[d]
Auxiliary protein	SecF homologue	#2.A.6.4.1.	IM	FN0700 (19704035)	FNV1362 (34763056)
	SecD homologue	#2.A.6.4.1.	IM	FN0699 (19704034)	FNV1361 (34763055)
	YajC homologue	#9.B.18.	IM	FN1335 (19704670)	FNV0699 (34763563)
	YidC homologue	#2.A.9.3.2.	IM	FN0004 (19703356)	FNV1558 (34763009)
	FtsY homologue	#3.A.5	C	FN1074 (19704409)	FNV1320 (34763139)
	Ffh homologue	#3.A.5	C	FN1393 (19704725)	FNV1070 (34763935)
Signal peptidase	SPase I		IM	FN0370 (19703712)	FNV0578 (34763844)
	SPase II		IM	FN0068 (19703420)	FNV0910 (34763704)
2. The Type 4 pilin/fimbriation secretion pathway		#3.A.15.2			
	PilD homologue		IM	FN2092 (19705382)	FNV1790 (34762867)
	PilC homologue	#3.A.15.2	IM	FN2094 (19705384)	FNV1792 (34762869)
	PilB homologue	#3.A.15.2	P	FN2095 (19705385)	FNV1793 (34762870)
	PilQ homologue	#1.B.22	OM	FN2086 (19705376)	FNV1783 (34762860)
	PilT homologue	#3.A.15.2	IM	FN1613 (19704934)	FNV2109 (34762538)
3. The holins		#1.E.			
	LrgA/CidA homologue	#1.E.14.	IM	FN0467 (19703802)	FNV2161 (34762272)
				FN1532 (19704864)	FNV1093 (34763425)
	LrgB/CidB homologue	#1.E.14.	IM	FN0468 (19703803)	FNV2160 (34762271)
				FN1531 (19704863)	
4. The large conductance mechanosensitive ion channel					
	MscL	#1.A.22	IM	FN0766 (19704101)	
5. The Type V secretion pathway					
The Type Va secretion pathway		#1.B.12			
	AT-1		OM/EC	FN1426 (19704758)	FNV0835 (34763535)
				FN1950 (19705252)	FNV2311 (34762138)
				FN0254 (19703599)	FNV2154 (34762265)[d]
				FN0387 (19703729)	FNV1681 (34762599)
				FN1449 (19704781)	FNV0939 (34763585)[d]
				FN1526 (19704858)	FNV0828 (34763560)
				FN1554 (19704886)	FNV2120 (34762230)
				FN1893 (19705198)	FNV2033 (34762372)[d]
				FN2047 (19705337)	FNV1037 (34763479)
				FN2058 (19705348)	FNV2094 (34762523)[d]
				FN1905 (19705210)	FNV1736 (34762834)[d]
				FN0498 (19703833)	
				FN1381 (19704716)	
The Type Vb secretion pathway		#1.B.20			
	TpsB		OM	FN0131 (19703476)	FNV1202 (34763328)
				FN0292 (19703637)[d]	FNV1408 (34762923)[d]
				FN1818 (19705123)	FNV0255 (34764256)[d]
				FN1911 (19705216)	
	TpsA		EC	FN0132 (19703477)[d]	FNV1203 (34763329)
				FN0291 (19703636)[d]	FNV1407 (34762922)
				FN0291 (19703636)	
				FN1817 (19705122)	
The Type Vc secretion pathway		#1.B.42			
	AT-2		OM/EC	FN0471 (19703806)	FNV1729 (34762484)
				FN0735 (19704070)	
				FN1499 (19704831)	

[a] TC: transport classification system.
[b] C: cytoplasm; EC: extracellular; IM: inner membrane; OM: outer membrane; P: peripheral to IM.
[c] GI: GenBank index number.
[d] Frameshifted sequence.

extracellular medium since it requires (i) a membrane fusion protein (MFP; TC #8.A.1), which spans the inner membrane and the periplasm, and even more importantly (ii) a channel-forming outer membrane factor TolC homologue (OMF; TC #1.B.17), which is absolutely necessary to complete the secretion [14,68]. However, neither PSI-BLAST nor TC-DB searches could identify an ABC protein exporter belonging either to the protein-1 exporter family (TC #3.A.1.109) or to the protein-2 exporter family (TC #3.A.1.110). Moreover, similar searches demonstrated that all MFP homologues (FN0516, FN0826 and FN1274) and OMF TolC homologues identified (FN1273, FN0517, FNV0260 and FNV0611) were systematically associated with detoxification efflux systems, and belong to the resistance-nodulation-cell division (RND) superfamily (TC #2.A.6) [68]. As a consequence, a Type I protein secretion system is likely absent from *F. nucleatum*.

Surprisingly, this genomic analysis revealed the presence of a locus resembling a Tfp system and several proteins resembling members of the autotransporters, TPS and YadA-like trimeric autotransporters family and, therefore, the presence of a complete range of Type V secretion system: the Type Va, Vb and Vc secretion pathways [24,25] (Table 1). The Type V secretion pathway can be briefly defined as the secretion of proteins that are translocated across the outer membrane via a transmembrane pore formed by a β-barrel and contain all the information required for translocation of an effector molecule through the cell envelope [21].

3.2. Type 4 pilus secretion pathway

To avoid any confusion between the GSP and the Type II secretion pathway [69], we prefer to use here the terminology based on the pullulanase (Pul) secretion in *Klebsiella oxytoca* to describe the components of the Type II secretion pathway [19]. Depending on the species, between 12 and 15 genes have been identified as being essential for Type II secretion [17]. Initial investigations reported that the Type II secretion pathway was missing in *F. nucleatum* subsp. *nucleatum* ATCC 25586 [3]. However, later studies reported it as incomplete in both *F. nucleatum* subsp. *nucleatum* ATCC 25586 and *F. nucleatum* subsp. *vincentii* ATCC 49256 [4] since only homologues of (i) the ATPase PulE, (ii) the multispanning transmembrane protein PulF, (iii) the secretin PulD and (iv) the prepseudopilin PulG were identified. Further analyses by Kapatral et al. did not reveal other proteins related to this protein secretion pathway which led these researchers to conclude the probable loss of the Type II secretion system in *F. nucleatum* [4]. The Type II secretory pathway is closely related to the secretion pathway involved in biogenesis of Tfp which are important for the virulence of many bacterial pathogens [19,70,71].

At least four proteins have proven to be homologous between the Type II and Tfp secretion pathways, i.e., (i) the ATPases PulE and PilB, (ii) the multispanning transmembrane proteins PulF and PilC, (iii) the secretins PulD and PilQ and (iv) the prepilin peptidases PulO and PilD [70,71]. Following PSI-BLAST and Pfam searches, we identified a PulO homologue (FN2092) possessing the Tfp leader peptidase domain (PF01478), and which was previously annotated as an integral membrane protein. These results prompted us to consider these protein components as part of a Tfp system rather than a defunct Type II secretion pathway since (i) from TC-DB searches, the proteins originally annotated PulE, PulF, PulD and PulG show stronger similarities with the Tfp system (TC #3.A.15.2; *E*-values $\leq 2\times10^{-74}$, $\leq 1\times10^{-25}$, $\leq 2\times10^{-21}$ and $\leq 2\times10^{-5}$, respectively) than with the pullulanase secretion system (TC #3.A.15.1), and (ii) the ATPase and multispanning transmembrane components cluster phylogenetically with the PilB ATPases (cluster 2) and the PilC integral membrane proteins (cluster 2) [70] (Table 1). Importantly, additional proteins are involved in the Tfp system but not in the Type II secretion system, such as the ATPases PilT implicated in pilus-generated movement termed twitching motility [19,72]. During the current investigation, we identified a protein originally annotated as hypothetical (FN1613), but which is homologous to the twitching mobility protein PilT from *Pseudomonas aeruginosa* (PA0395; GI: 15595592) as indicated by (i) 36% identity and 57% similarity, (ii) TC-DB searches (*E*-values $\leq 1\times10^{-48}$), as well as (iii) phylogenetic clustering of these proteins within the ATPase cluster 2 (Table 1; see also supplementary materials) [70]. Except for *pilT,* which is located elsewhere on the chromosome, *pilQ*, *pilD*, *pilA*, *pilC* and *pilB* are present in the same locus (Fig. 1A). The five ORFs present between *pilQ* and *pilD* were originally annotated as encoding hypothetical proteins; a closer look at the nucleotide sequence revealed the start codon of the three ORFs, i.e., FN2089, FN2091 and FN2093, were misannotated and are more likely to be present further upstream (see supplementary material). Following CDD search, FN2088 shows homology with PilN (*E*-value$=2.0\times10^{-4}$), but no significant homology could be found for the other ORFs following PSI-BLAST, Pfam, InterProScan or CDD searches. However, when aligned with known major (PilA) and minor (PilE, PilV, PilW, PilX, FimT and FimU) Tfp prepilins from *Pseudomonas aeruginosa* PA01 [72,73], the N-terminal region of FN2089, FN2090 and FN2091 display typical Tfp prepilin signal sequences (Fig. 1B) including (i) a hydrophobic N-terminal α-helical region, (ii) a consensus (K/R)(G/A)↓(F/Y/Hy) motif present between the n- and h-domain of the signal peptide and (iii) a glutamine residue present at position +5 of the cleavage site [72,73]. Therefore, these putative proteins could be substrates of the Tfp prepilin peptidase PilD and with PilA compose a fusobacterial Tfp.

3.3. The Type Va protein secretion pathway

The Type Va secretion pathway, or the classical autotransporter protein secretion system (AT-1; TC #1.B.12), was first described for the IgA1 protease

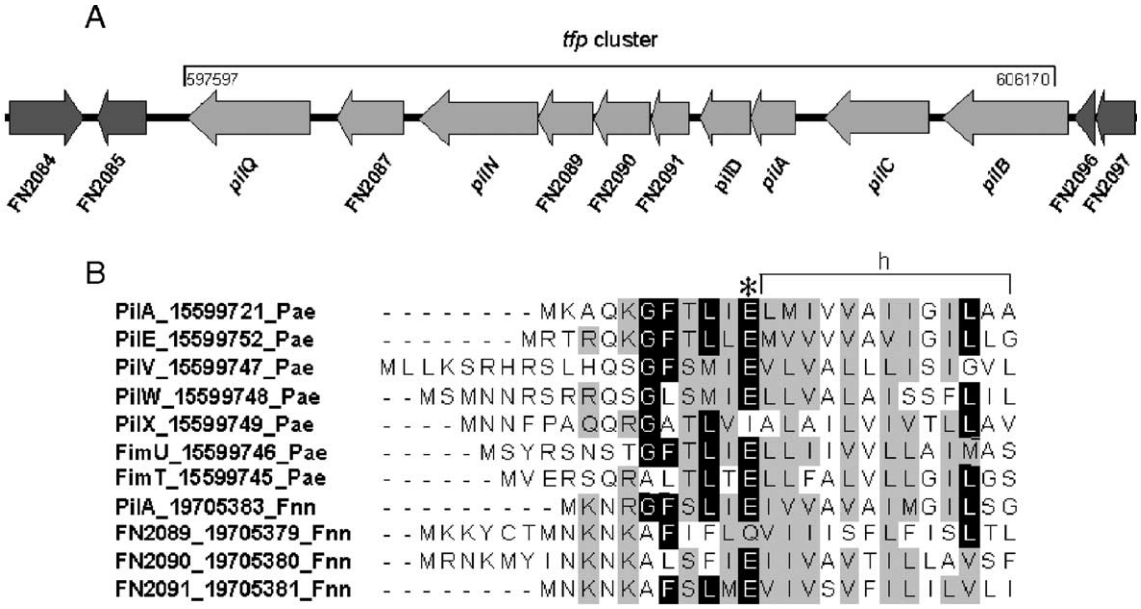

Fig. 1. The putative *tfp* locus. (A) The putative *F. nucleatum* subsp. *nucleatum* ATCC 25586 tfp locus is depicted. Light shading indicates the genes encoding each putative Tfp component; genes outside the putative *tfp* locus are indicated by darker shading. The genome coordinates of the *tfp* locus are indicated, as are the identities of each gene. (B) Alignment of the N-terminal signal sequences of the putative Tfp prepilins found in *F. nucleatum* subsp. *nucleatum* ATCC 25586 (Fnn) with the known and characterised prepilins from *P. aeruginosa* PA01 (Pae). Like *P. aeruginosa*, the *F. nucleatum* prepilins demonstrate a conserved glutamine residue (*) followed by a hydrophobic segment (h).

produced by *Neisseria gonorrhoeae* [74]. In essence, an autotransporter is a modular protein synthesised as a single polypeptide and composed from its N- to C-terminus of (i) a signal peptide, (ii) a passenger domain corresponding to the effector molecule and (iii) a translocation unit composed of a short linker region having an α-helical secondary structure and a β-domain that will adopt a β-barrel secondary structure when embedded in the outer membrane.

Iterative PSI-BLAST searches, using the translocation unit of representatives of each of the 34 deep-rooted phylogenetic groups of autotransporters as queries (Fig. 2), identified 24 putative autotransporter proteins in the two *F. nucleatum* species investigated; 13 in subsp. *nucleatum* and 11 in subsp. *vincentii* (Table 2). In all cases, Pfam searches revealed the presence of a C-terminal domain characteristic of autotransporter β-domains (PF03797; *E*-values $\leq 5.6\times10^{-34}$). Analyses of the secondary structure of the C-terminal part of these proteins using AMPHI, PSIPRED and PRED-TMBB predicted that they all possess typical translocation units composed of an α-helix and a β-barrel containing around 14 β-strands (see supplementary materials).

As mentioned previously, translocation of autotransporter proteins through the inner membrane occurs via the Sec apparatus [21,22]. Using SignalP, PSORT and LipoP trained on Gram-negative and Gram-positive bacteria, none of these potential autotransporters were predicted to possess signal sequences recognised by signal peptidase I or signal peptidase II. However, using PSORTb for computational prediction of the subcellular localisation of proteins, and in agreement with the outer membrane β-barrel structures predicted above, all the potential fusobacterial autotransporters were predicted to be located in the outer membrane. However, closer inspection of the nucleotide sequences revealed that the start codon of all these putative autotransporters had been misannotated (Table 2; see also supplementary material). Indeed, for *F. nucleatum* subsp. *nucleatum,* a putative ribosome-binding site and a start codon was found in-frame further upstream in all genes encoding autotransporter proteins. In light of this new information, analyses of these genes revealed the capacity of these genes to encode proteins ranging from 930 to 3738 amino acids, with predicted signal peptides ranging from 20 to 41 amino acids, sizes which are in accordance with normal autotransporter proteins and signal sequences, respectively (Table 2).

While ten orthologues could be identified between *F. nucleatum* subsp. *nucleatum* and *F. nucleatum* subsp. *vincentii* following BLAST and PSI-BLAST searches, it also appeared that FNV0939, FNV2154, FNV2033, FNV2094 and FNV1736 from *F. nucleatum* subsp. *vincentii* are frameshifted when compared with their respective orthologue FN0254, FN1526, FN1554, FN1905 and FN0498 from *F. nucleatum* subsp. *nucleatum* (Table 2; see also supplementary material); however, it is impossible to make a qualitative judgement about the state of the genes encoding these autotransporter proteins as the *F. nucleatum* subsp. *vincentii* genome is incomplete. Interestingly, while no orthologue of FNV1037 could be found in *F. nucleatum* subsp. *nucleatum*, sequence alignments revealed that the N- and C-terminal parts of the protein, including the signal sequence and the translocation

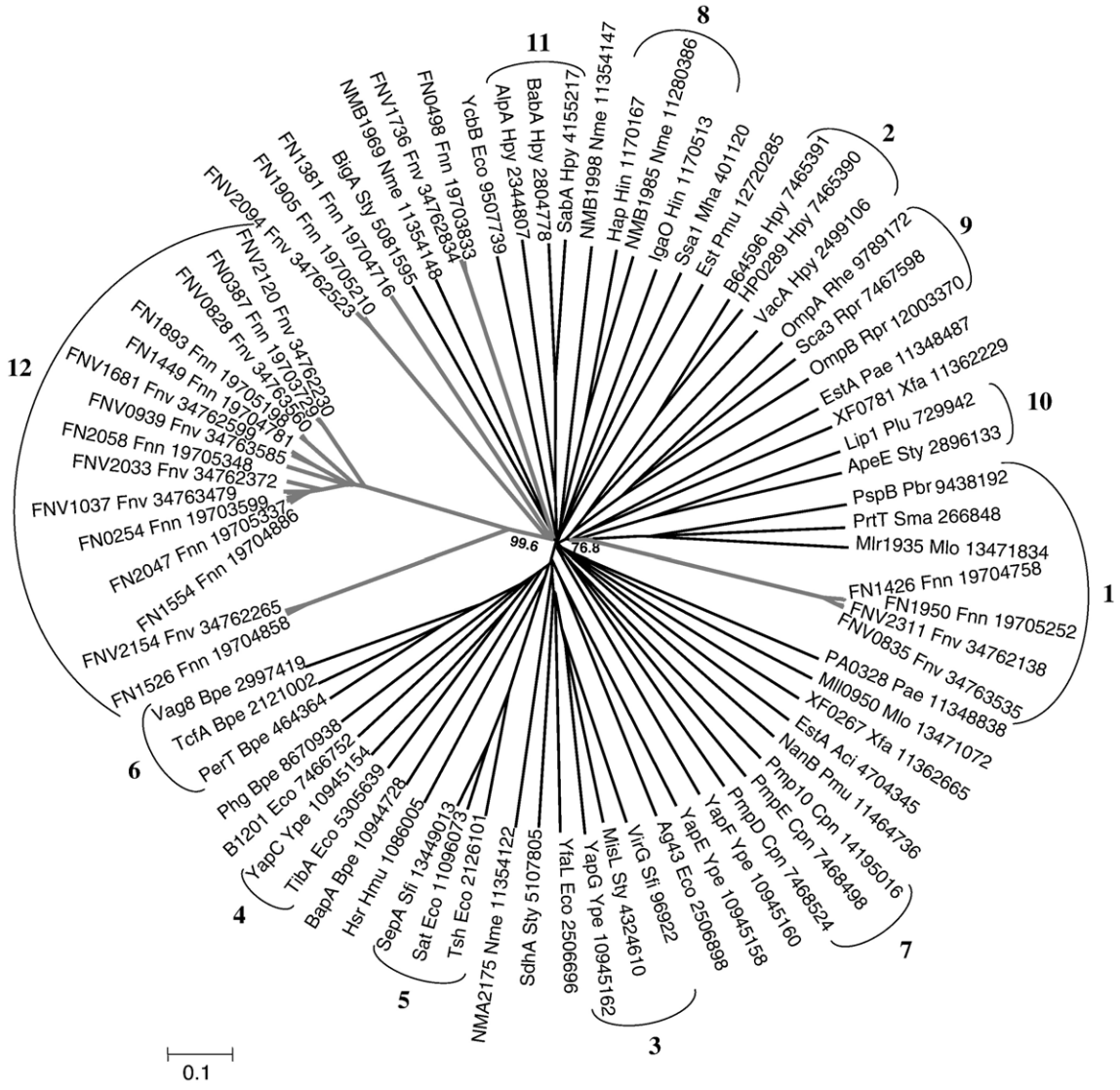

Fig. 2. Phylogenetic tree of currently recognised members of the autotransporter family in relation to the newly found autotransporters in *F. nucleatum* sp. *nucleatum* and *F. nucleatum* sp. *vincentii*. The tree is derived from analyses of the C-terminal translocation unit of autotransporters. The scale bar represents the evolutionary distance, i.e., the number of substitutions per site. The values on branches before nodes are the bootstrap confidence levels expressed in percentage. Bootstraps are given for clusters containing fusobacterial proteins only. Branches containing fusobacterial protein(s) are greyed. Aci: *Acidiphilium* sp. AIU409; Bpe: *Bordetella pertussis*; Cpn: *Chlamydophila pneumoniae*; Ype: *Yersinia pestis*; Eco: *Escherichia coli*; Fnn: *Fusobacterium nucleatum* sp. *nucleatum*; Fnv: *Fusobacterium nucleatum* sp. *vincentii*; Hin: *Haemophilus influenzae*; Hmu: *Helicobacter mustelae*; Hpy: *Helicobacter pylori*; Mha: *Mannheimia haemolytica*; Mlo: *Mesorhizobium loti*; Nme: *Neisseria meningitidis*; Pae: *Pseudomonas aeruginosa*; Pbr: *Pseudomonas brassicacearum*; Plu: *Photorhabdus luminescens*; Pmu: *Pasteurella multocida*; Rhe: *Rickettsia helvetica*; Rpr: *Rickettsia prowazekii*; Sfl: *Shigella flexneri*; Sma: *Serratia marcescens*; Sty: *Salmonella typhimurium*; Xfa: *Xylella fastidiosa*.

unit, respectively, are highly similar to those found in FN0254, FN0387, FN1449, FN1554, FN1893, FN2047 and FN2058; however, the central region of these proteins, corresponding to the functional passenger domain, is much more divergent (see supplementary material). The same observation applied for FN1426 and FNV0835 which both share high similarity with FN1950 and FNV2311 in their N- and C-terminal regions.

A recent investigation also revealed the presence in several autotransporters of a conserved intramolecular chaperone domain (PD002475) located upstream of the translocation unit and involved in the surface folding of the passenger domain as it emerges from the β-domain channel [75]. However, ProDom searches could not detect the presence of the autochaperone domain; this domain is not systematically present in the autotransporters indicating either that distinct types of endochaperone are present in various autotransporters and/or this domain is not a general feature of autotransporters [75].

The only solved structure of a secreted autotransporter passenger domain, i.e., P.69 pertactin from *Bordetella pertussis*, shows a parallel β-helical structure terminating in the putative autochaperone domain [76]. It was proposed that ongoing folding of this β-roll structure might pull the

Table 2
Autotransporter proteins of *F. nucleatum* subsp. *nucleatum* ATCC 25586

Locus name	Orthologue[a]	Length[b]	Signal sequence[c]	BetaWrap[d]	Annotation	Significant functional motif[e]
FN1426	FNV0835 (67%)	1016	28 (GGG-GG)	487–633 (3.9×10^{-3})	Serine protease	Subtilisin-like peptidase S8A (IPR011165)
FN1950	FNV2311 (89%)	1023	28 (SGS-GG)	447–559 (1.3×10^{-2})	Serine protease	Subtilisin-like peptidase S8A (IPR011165)
FN0254	FNV0939 (–)[f]	2105	42 (AFS-EE)	756–868 (1.4×10^{-5})	*Fusobacterium* outer membrane protein family	RGD cell-attachment motif (PS00016)
FN0387	FNV2120 (92%)	2214	41 (VFS-EE)	833–951 (3.9×10^{-4})	*Fusobacterium* outer membrane protein family	RGD cell-attachment motif (PS00016)
FN1449	FNV1681 (81%)	3738	41 (AFS-EE)	1094–1202 (5.2×10^{-4})	*Fusobacterium* outer membrane protein family	Cysteine peptidase active site (PS00639); Filamin repeats (PS50194); Spectrin repeat (IPR002017)
FN1526	FNV2154 (–)[f]	3472	40 (SYA-DE)	2437–2596 (5.6×10^{-4})	*Fusobacterium* outer membrane protein family	RGD cell-attachment motif (PS00016); ATP- and GTP-binding site motif A (P-loop) (PS00017)
FN1554	FNV2033 (–)[f]	2361	41 (AFS-EE)	1272–1415 (3.7×10^{-3})	*Fusobacterium* outer membrane protein family	ATP- and GTP-binding site motif A (P-loop) (PS00017)
FN1893	FNV0828 (93%)	2368	41 (AFS-EE)	944–1091 (6.7×10^{-3})	*Fusobacterium* outer membrane protein family	RGD cell-attachment motif (PS00016); Invasin/intimin cell adhesion motif (IPR008964)
FN1905	FNV2094 (–)[f]	1500	20 (SYS-ED)	1309–1428 (9.8×10^{-3})	168 kDa surface-layer protein precursor	None
FN0498	FNV1736 (–)[f]	1536	20 (SYA-DE)	1239–1347 (4.9×10^{-2})	Unknown	None
FN2047	–	2429	42 (AFS-EE)	1327–1443 (1.2×10^{-4})	*Fusobacterium* outer membrane protein family	RGD cell-attachment motif (PS00016)
FN2058	–	2381	42 (AFS-EE)	1242–1371 (5.7×10^{-4})	*Fusobacterium* outer membrane protein family	RGD cell-attachment motif (PS00016); ATP- and GTP-binding site motif A (P-loop) (PS00017)
FN1381	–	1231	21 (AYG-GW)	135–265 (1.0×10^{-3})	Unknown	ATP- and GTP-binding site motif A (P-loop) (PS00017)
–	FNV1037	1946	42 (AFS-EE)	1082–1215 (6.4×10^{-3})	*Fusobacterium* outer membrane protein family	RGD cell-attachment motif (PS00016); ATP- and GTP-binding site motif A (P-loop) (PS00017)

[a] Orthologues present in *F. nucleatum* subsp. *vincentii* ATCC 49256; percentage of similarity is indicated in parentheses whenever possible.
[b] Protein length expressed in number of amino acids including the signal peptide.
[c] Results from SignalP, PSORT and/or LipoP searches where the length of the signal peptide is given in number of amino acids and the cleavage site is given in parentheses.
[d] Position of the best wrap calculated by BetaWrap with the *P* value given in parentheses.
[e] Functional motifs were found using ScanProsite and/or InterProScan.
[f] Frameshifted sequences; for these sequences length, presence of signal sequence, BetaWrap prediction and the presence of functional motif are not indicated.

passenger domain through the pore and provide the energy for translocation [77]. The recently developed program named BetaWrap can predict parallel β-helices from the amino acid sequence [48,49]. This program predicts wraps formed by a packet of five rungs: (i) a single rung has three strands (B1, B2 and B3) separated by three turns (T1, T2 and T3), (ii) while the lengths of T1 and T3 vary, T2 is almost always 2 residues long, (iii) aliphatic residues in T2 facilitate the stacking of the rungs, (iv) the start position of B1 and B2 describe the position of the rung. Comprehensive analyses revealed that such parallel β-helical structure was present in the majority of autotransporter passenger domains [78,79]. Using BetaWrap to assess the potential for the putative fusobacterial autotransporters to fold into a β-helical structure, such secondary structure was detected in all passenger domains (Table 2).

As mentioned previously, the passenger domains represent the effector molecules of autotransporter proteins. Except for the four putative autotransporters originally annotated as serine proteases, which possess subtilase-like peptidase domains (IPR011165; E-values $\leq 1.0\times10^{-30}$) (Table 2), all other autotransporters were annotated as outer membrane proteins with no other indication on their function. Thus, Pfam, InterProScan, CDD and ScanProsite

searches were combined to identify significant functional motifs within the newly identified full-length passenger domains. In FN1449, a signature for a cysteine peptidase active site, spectrin repeats (IPR002017; *E*-values $\leq 3.4\times10^{-3}$) and filamin repeat motifs (PS50194) were identified (Table 2). Invasin/intimin cell adhesion motifs (IPR008964; *E*-values $\leq 1.9\times10^{-2}$) were identified in FN1893. In association with invasin/intimin cell adhesion motifs or alone, RGD cell-attachment motifs (PS00016) were found in several other passenger domains (Table 2). Several ATP- and GTP-binding site motifs (PS00017) were also identified in the passenger domain of fusobacterial autotransporters (Table 2); the presence of such motifs was previously reported in other autotransporters but a definitive function has not been attributed to these motifs [21]. In two proteins, i.e., FN0498 and FN1905, no significant functional motif could be found.

Previous phylogenetic analyses based on the translocation unit of autotransporters revealed that microorganisms possessing autotransporters are found within the phyla *Proteobacteria* and *Chlamydiae* [23,30,80,81]. Phylogenetic analyses of the newly found fusobacterial autotransporters reveals 4 additional deep-rooted branches, one of them displaying a novel protein clustering, i.e., cluster 12 (Fig. 2). Furthermore, the fusobacterial autotransporters originally annotated as serine proteases are grouped with autotransporters of cluster 1; this cluster was formerly described as containing γ1, γ3 and α-proteobacterial subtilisin proteases [80]. Therefore, phylogenetic analyses including the fusobacterial autotransporter translocation units reveal a tree organised around 38 deep-rooted branches including 12 clusters (Fig. 2).

3.4. The Type Vb secretion pathway

The two-partner secretion system (TPS; TC #1.B.20), or Type Vb secretion pathway, was first characterised for the ShlA/ShlB hemolysin system of *Serratia marcescens*, in which ShlB forms a β-barrel pore in the outer membrane allowing the translocation of the hemolysin ShlA from the periplasm to the extracellular milieu [82]. In contrast to the Type Va pathway where the autotransporter protein is produced as a single polypeptide, in the Type Vb pathway the effector molecule (also called the exoprotein or TpsA) and the pore forming β-domain, (also called the transporter protein or TpsB) are translated as two distinct proteins [83]. Still, each TpsB transporter described so far is dedicated to the secretion of only one TpsA exoprotein [83].

After three iterations using the TpsB protein FhaC from *Bordetella pertussis* (BP1884; 33592931) as a query, PSI-BLAST searches (*E*-values $\leq 5.0\times10^{-55}$) revealed the presence of four genes encoding putative TpsB proteins in *F. nucleatum* subsp. *nucleatum* (FN0131, FN0292, FN1818 and FN1911; Table 3). Further sequence alignments also revealed FN0131 and FN1818 are paralogous, but only one orthologue of each of FN0131, FN0292 and FN1911 was found in *F. nucleatum* subsp. *vincentii* viz. FNV1408, FNV1202 and FNV0254, respectively (Table 3; see also supplementary material).

Table 3
TpsB proteins of *F. nucleatum* subsp. *nucleatum* ATCC 25586

Locus name	Orthologue[a]	Length[b]	Signal sequence[c]	Annotation
FN0131	FNV1408 (–)[d]	566	16 (VFA-FS)	Hemolysin activator protein precursor
FN1818[d]	–	–	–	Hemolysin activator protein precursor
FN0292[d]	FNV1202 (–)[d]	–	–	Hemolysin activator protein precursor
FN1911	FNV0254 (–)[d]	697	21 (TMV-NL)	Outer membrane protein

[a] Orthologues present in *F. nucleatum* subsp. *vincentii* ATCC 49256; percentage of similarity is indicated in parentheses whenever possible.
[b] Protein length expressed in number of amino acids including the signal peptide.
[c] Results from SignalP, PSORT and/or LipoP searches where the length of the signal peptide is given in number of amino acids and the cleavage site is given in parentheses.
[d] Frameshifted sequence; for these sequences length and presence of signal sequence are not indicated.

As with the autotransporters, TpsB reaches the periplasmic space after translocation through the Sec translocon. However, LipoP, PSORT and SignalP trained on Gram-negative and Gram-positive bacteria could predict the presence of a signal in only one putative *Fusobacterium nucleatum* subsp. *nucleatum* TpsB protein, i.e., FN0131. Closer inspection of the nucleotide sequence of the paralogous FN1818 revealed a signal sequence is encoded in a different frame to the C-terminal portion of the protein (Table 4). The presence of a poly-A nucleotide stretch suggests that expression of this TpsB protein may be phase variable in which translational slipped-strand mispairing occurs. Interestingly, the signal sequence of FN0292 is also encoded in a different reading frame and, based on the presence of a poly-AT stretch, also appears to possess the ability to undergo phase variation by slipped-strand mispairing (see supplementary material). No signal peptide could be originally predicted in FN1911, however, closer inspection of the open-reading frame revealed that the start codon was misannotated and in fact occurred in-frame further upstream; the newly identified full-length protein possesses a typical signal sequence (Table 3; see supplementary material).

Examination of the full-length proteins revealed that all of the putative fusobacterial TpsB proteins exhibit a typical translocation unit composed of an α-helix and a β-barrel containing 16–19 β-strands as predicted by PSIPRED and PRED-TMBB, respectively (see also supplementary materials). Pfam searches (*E*-values $\leq 7.0\times10^{-10}$) of the full-length *F. nucleatum* subsp. *nucleatum* TpsB proteins

Table 4
DNA region encoding the signal peptides of TpsB proteins in *F. nucleatum* subsp. *nucleatum* ATCC 25586

Locus name	DNA sequence translation[a]	Signal peptide[b]
FN0131	TAATATATAAATTAATAGTATGAGAGGAGGAGTATGAAAAAAATAGGAACATATATTTTTCTGGTTTTTAATGTTTTTGCTTTTTTCAGATTCTTTTAAT	
	+1: **M K K K I G T Y I F L V F N V F A F S D S F N**	18 (VFA-FS)
FN1818	TAATATATAAATTAATAGTATGAGAGGAGGAGTATGAAAAAATAGTAACATATATTTTTCTGGTTTTTAATGTTTTTGCTTTTTTCAGATTCTTTTAAT	
	+1: **M K K K** * * H I F F W F L M F L L F Q I L L	18 (VFA-FS)
	+2: * K N S N I Y F S G F * C F C F F * * E *	
	+3: E K I V T Y I F L V F N V F A F S D S F N	

[a] Putative ribosome binding sites are underlined; potentially functional signal sequence are indicated in bold; potential nucleotide sequence responsible for frameshift are indicated in red.
[b] SignalP results given for the potentially functional signal sequence only.

revealed the presence of a hemolysin activator domain (PF03865) within FN0131 and FN1818 further supporting a role for these proteins in the Type Vb secretion pathway. Further analyses revealed that the full-length FN1911 exhibits a significant match with PF01103 (*E*-value= 1.8×10^{-71}) which is characteristic of surface antigens such as D15 from *Haemophilus influenzae*, Oma87 from *Pasteurella multocida* and Omp85 from *Neisseria meningitidis* and which represent a subset of the TpsB proteins which are implicated in outer membrane biogenesis. Moreover, the conserved RGY/F motif, with the consensus sequence (D/E)XHyXHyGGXX(S/T)HyRG(Y/F), found in a predicted surface loop located four transmembrane segments before the C-terminal residue of TpsB proteins was systematically present with at least two of the last three residues conserved (Fig. 3) [80]. It was recently hypothesised that this motif could be involved in the recognition of TpsA at some stage in the translocation process [84].

Considering that the genes encoding TpsA and TpsB proteins are generally organised in an operon, DNA regions in proximity of TpsB genes were inspected. With the exception of the Omp85-like FN1911 protein, TpsA proteins were found immediately adjacent to the genes encoding the TpsB proteins (Table 5). PSI-BLAST searches and further sequence alignments revealed TpsA proteins FN1817 and FN0132 are paralogous, but only one orthologue of each of FN1817 and FN0291 was found in *F. nucleatum* subsp. *vincentii* viz. FNV1407 and FNV1203 (Table 5; see also supplementary material). Closer inspection of the nucleotide sequence of the paralogous FN0132 revealed that a signal sequence is encoded in a different reading frame and, based on the presence of a poly-AT stretch, also appears to possess the ability to undergo phase variation by slipped-strand mispairing (see supplementary material). When compared with its orthologue FNV1203, the sequence of FN0291 appears to be frameshifted in its N-terminal region (see supplementary material). Therefore, only FN0291 and FN1817 could be potentially expressed (Table 5).

Since there is no covalent link between TpsB and TpsA, a specific recognition event must occur at the periplasmic side of the outer membrane prior to translocation of TpsA to the bacterial cell surface [84]. TpsA proteins bear a conserved and highly distinctive N-proximal domain called the TPS domain which is essential for secretion and is hypothesised to interact specifically with TpsB to initiate translocation. A TPS domain is systematically found in the N-proximal region of the putative fusobacterial TpsA proteins (Fig. 4). Interestingly, it has been proposed that the conserved TPS domain may serve the same function as the autochaperone domain found in autotransporters [84,85]. As found in the passenger domains of autotransporter proteins, a β-helix structure has been recently reported for the TpsA filamentous haemagglutinin (BP1879; 33592927) from *Bordetella petussis* [85]. Anal-

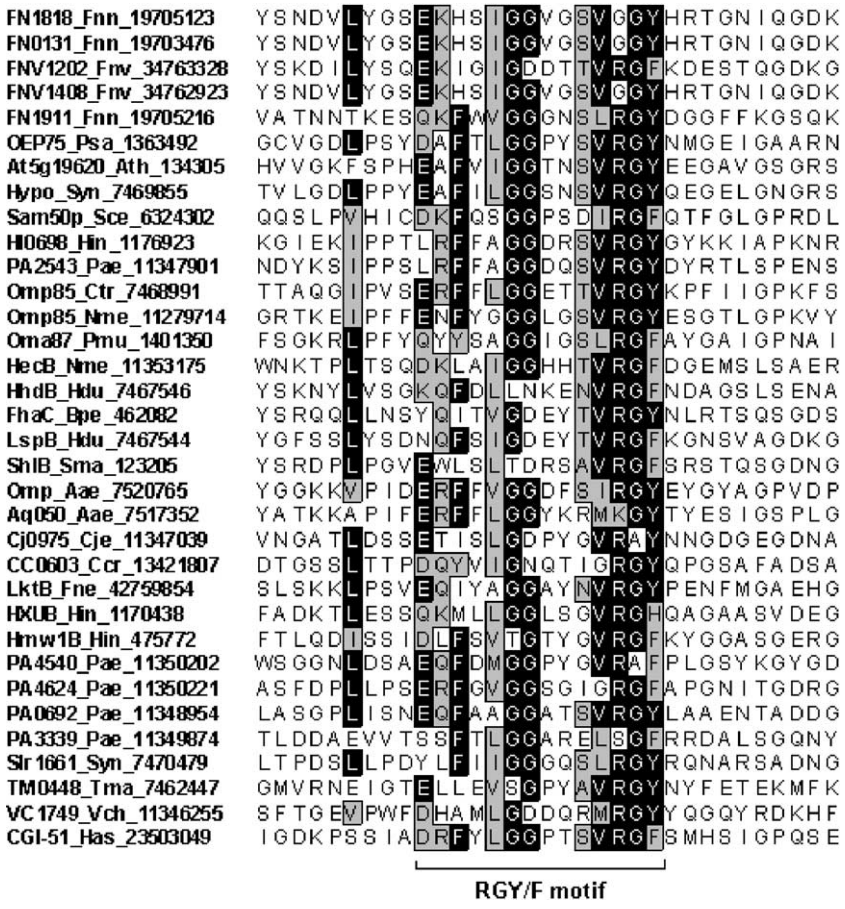

Fig. 3. Sequence alignment of fusobacterial TpsB and representative TpsB proteins from other species which harbor a conserved RGY/F motif. This motif is systematically present in the predicted surface loop located four transmembrane segments before the C-terminal residue. Aae: *Aquifex aeolicus*; Ath: *Arabidopsis thaliana*; Bpe: *Bordetella pertussis*; Ccr: *Caulobacter crescentus*; Cje: *Campylobacter jejuni*; Ctr: *Chlamydia trachomatis*; Fne: *Fusobacterium necrophorum*; Fnn: *Fusobacterium nucleatum* sp. *nucleatum*; Fnv: *Fusobacterium nucleatum* sp. *vincentii*; Has: *Homo sapiens*; Hdu: *Haemophilus ducreyi*; Hin: *Haemophilus influenzae*; Nme: *Neisseria meningitidis*; Pae: *Pseudomonas aeruginosa*; Pmu: *Pasteurella multocida*; Psa: *Pisum sativum*; Sce: *Saccharomyces cerevisae*; Sma: *Serratia marcescens*; Syn: *Synechocystis* sp.; Tma: *Thermotoga maritima*; Vch: *Vibrio cholerae*.

yses of the fusobacterial TpsA proteins with BetaWrap predicted similar secondary structure in all these exoproteins (Table 5). All TpsA proteins were annotated as hemolysins and bear haemagglutinin repeats (PF05594; E-values $\leq 1.4\times10^{-3}$), as well as haemagglutination activity domains (PF05860; E-values $\leq 4.1\times10^{-45}$).

Recent phylogenetic investigations of the Type Vb secretion system using TpsB proteins has revealed 20

Table 5
TpsA proteins of *F. nucleatum* subsp. *nucleatum* ATCC 25586

Locus name	Orthologue[a]	Length[b]	Signal sequence[c]	BetaWrap[d]	Annotation	Significant functional motif[e]
F. nucleatum sub. spp. *nucleatum* ATCC 5586						
FN0291[f]	FNV1203 (–)	–	–	–	Hemolysin	–
FN1817	FNV1407 (75%)	2806	24 (IFA-AN)	2115–2258 (4.3×10^{-4})	Hemolysin	Haemagglutination activity domain (PF05860); Haemagglutinin repeats (PF05594)
FN0132[f]	–	–	–	–	Hemolysin	–

[a] Percentage of similarity is indicated in parentheses whenever possible.
[b] Protein length expressed in number of amino acids including the signal peptide.
[c] Results from SignalP, PSORT and/or LipoP searches where the length of the signal peptide is given in number of amino acids and the cleavage site is given in parentheses.
[d] Position of the best wrap calculated by BetaWrap with the P value given in parentheses.
[e] Functional motifs were found using Pfam.
[f] Frameshifted sequence; for these sequences length, presence of signal sequence, BetaWrap prediction and the presence of functional motif are not indicated.

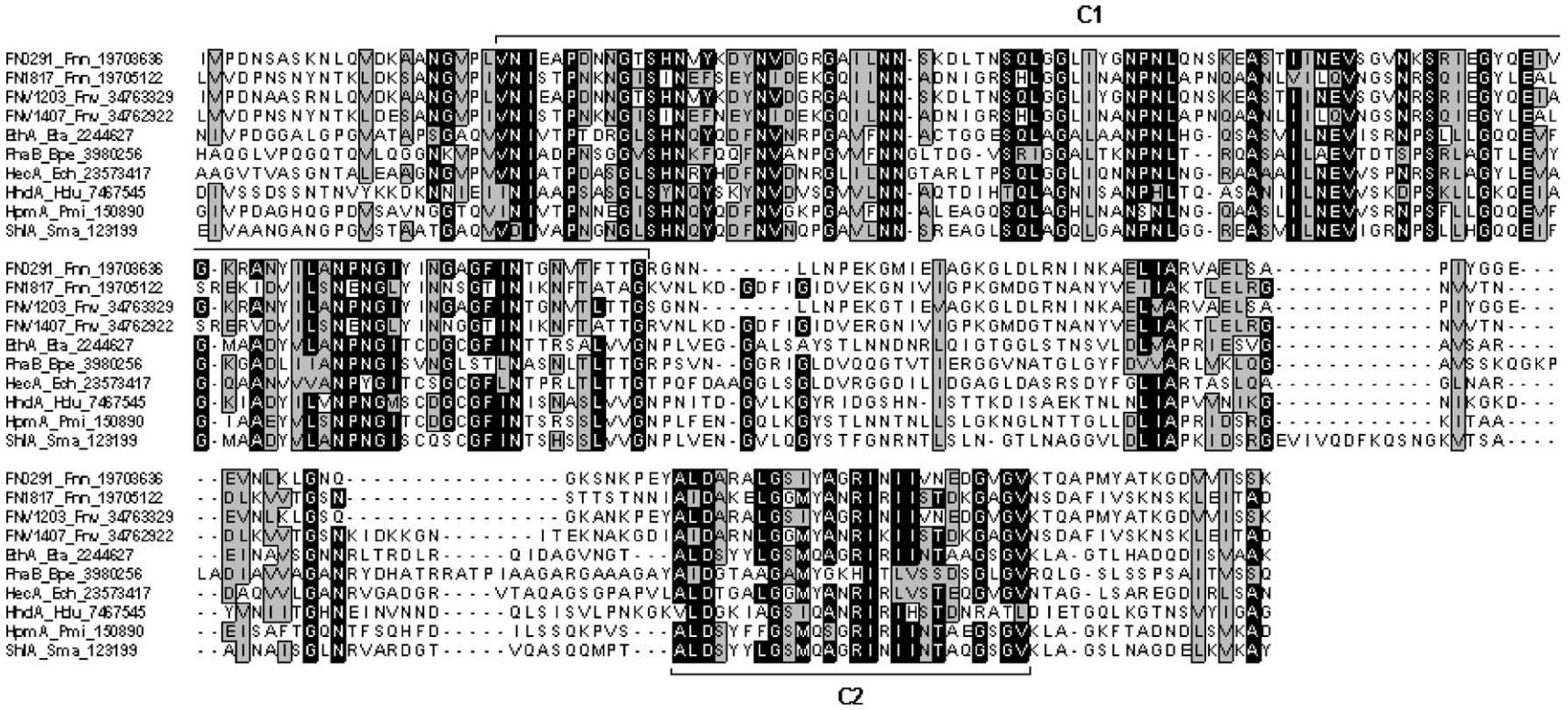

Fig. 4. Sequence alignment of the TPS domains of fusobacterial TpsA and representative TpsA proteins from other species. C1 and C2 represent two regions of high conservation in the TPS domains [84]. Bpe: *Bordetella pertussis*; Ech: *Erwinia chrysanthemi*; Eta: *Edwardsiella tarda*; Fnn: *Fusobacterium nucleatum* sp. *nucleatum*; Fnv: *Fusobacterium nucleatum* sp. *vincentii*; Hdu: *Haemophilus ducrey*; Hin: *Haemophilus influenzae*; Pmi: *Proteus mirabilis*; Sma: *Serratia marcescens*.

deeply rooted branches with six of them forming clusters of several proteins. Phylogenetic analyses using the translocation unit of the newly found putative TpsB proteins of *F. nucleatum* revealed that most of them cluster within group 6 (Fig. 5). Previous investigations suggested that this cluster exclusively contained proteobacterial proteins whilst this current study indicates this previous assumption is incorrect. Interestingly, essential outer membrane proteins shown to be involved in outer membrane biogenesis, i.e., Omp85-like proteins, have been included within the TpsB family, i.e., clusters 4 and 5 [80]. No organism encodes more than one representative protein in these clusters. The Omp85-like protein present in *F. nucleatum* subsp. *nucleatum*, i.e., FN1911, was not found within cluster 5 but on a new deeply rooted branch (Fig. 5). It should be mentioned that Omp85-like proteins from *Chlamydiae* also cluster on independent branches [80]. As a result the TpsB phylogenetic tree now appears to be formed of 21 deep-rooted branches with 6 of them displaying protein clustering.

3.5. The Type Vc secretion pathway

Based on analyses of YadA from *Yersinia enterocolitica* and Hia from *Haemophilus influenzae* members of the Type

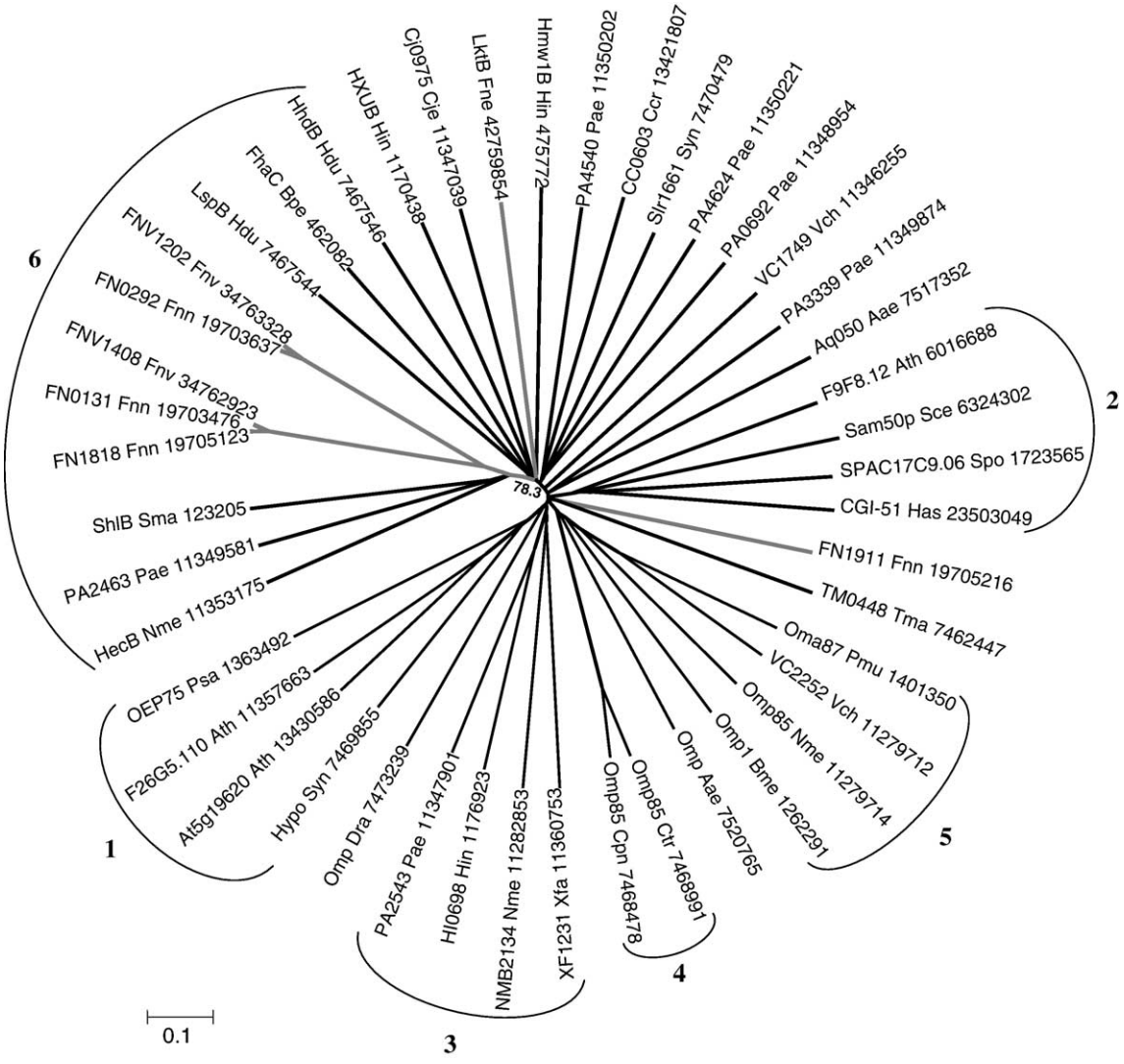

Fig. 5. Phylogenetic tree of currently recognised members of TpsB family in relation to the newly found TpsB proteins in *F. nucleatum* sp. *nucleatum* and *F. nucleatum* sp. *vincentii*. The scale bar represents the evolutionary distance, i.e., the number of substitutions per site. The values on branches before nodes are the bootstrap confidence levels expressed in percentage. Bootstraps are given for clusters containing fusobacterial proteins only. Branches containing fusobacterial protein(s) are greyed. Aae: *Aquifex aeolicus*; Ath: *Arabidopsis thaliana*; Bme: *Brucella melitensis*; Bpe: *Bordetella pertussis*; Ccr: *Caulobacter crescentus*; Cje: *Campylobacter jejuni*; Cpn: *Chlamydophila pneumoniae*; Ctr: *Chlamydia trachomatis*; Dra: *Deinococcus radiodurans*; Fne: *Fusobacterium necrophorum*; Fnn: *Fusobacterium nucleatum* sp. *nucleatum*; Fnv: *Fusobacterium nucleatum* sp. *vincentii*; Has: *Homo sapiens*; Hdu: *Haemophilus ducreyi*; Hin: *Haemophilus influenzae*; Hpy: *Helicobacter pylori*; Nme: *Neisseria meningitidis*; Pae: *Pseudomonas aeruginosa*; Pmu: *Pasteurella multocida*; Psa: *Pisum sativum*; Sce: *Saccharomyces cerevisae*; Sma: *Serratia marcescens*; Spo: *Schizosaccharomyces pombe*; Syn: *Synechocystis* sp.; Tma: *Thermotoga maritima*; Vch: *Vibrio cholerae*; Xfa: *Xylella fastidiosa*.

Vc protein secretion family (AT-2, TC#1.B.42; http://www.biology.ucsd.edu/~msaier/transport/) have recently been described as a subfamily of surface-attached oligomeric autotransporters [86]. Like the classical autotransporters, after translocation through the inner membrane via the Sec apparatus, the AT-2 proteins are displayed on the bacterial cell surface and anchored in the outer membrane by their C-termini [87]; deletion of the C-terminal domain abolishes outer membrane insertion while deletion of the linker region results in degradation of the whole protein. In contrast to the autotransporters the C-terminal region is proposed to form a β-barrel pore consisting of 12 β-strands only after trimerisation has occurred.

Following PSI-BLAST searches using the C-terminal region of YadA (GI: 32470319) as a query, after three iterations three putative AT-2 proteins (FN0471, FN0735, FN1499; *E*-value $\leq 10^{-6}$) were found in *F. nucleatum* subsp. *nucleatum* (Table 6). While FN1499 from *F. nucleatum* subsp. *nucleatum* is orthologous to FNV1729 from *F. nucleatum* subsp. *vincentii*, no orthologue to FN0471 and FN0735 from *F. nucleatum* subsp. *nucleatum* could be found in *F. nucleatum* subsp. *vincentii* though completion of the genome may reveal orthologues at a later date. It appears the start codon of both FN0471 and FN0735 were misannotated; in FN0471, a start codon was found in-frame further upstream, while in the case of FN0735, it was found in-frame further downstream, with a putative ribosome-binding site present in both cases upstream of the newly predicted start codon (see supplementary material). Interestingly, when comparing the amino acid sequence of FN1499 with FN0471 and FN0735, it appears that the N- and C-terminal parts of the proteins are highly similar but an additional sequence is present in the passenger domain of FN1499; in the same way, an additional sequence is present in the passenger domain of FN0735 when compared to FN0471 (see supplementary material). These proteins display (i) typical signal peptides, and (ii) typical translocation units consisting of an α-helix and four β-strands as predicted by PSIPRED and PRED-TMBB, respectively (Table 6; see also supplementary materials). Furthermore, these three proteins, which were originally annotated as cell surface proteins, (i) share homology with the Hia autotransporter adhesin (COG5295.1) at their C-terminus as revealed by CDD searches (*E*-values $\leq 6\times10^{-14}$), (ii) possess haemagglutinin motifs (PF05662) and invasin repeats (PF05658) as revealed by Pfam searches (*E*-values $\leq 1.9\times10^{-5}$ and $\leq 4.2\times10^{-4}$, respectively), and (iii) exhibit a β-helical structure in their passenger domain (Table 6).

Recent structural investigations revealed that the β-helical structure in the passenger domains of YadA-like trimeric autotransporters forms coiled-coil oligomers [88]. With the exception of FN0471 where the probability is low, sequence analysis with COILS showed that the passenger domains of these proteins display extended coiled-coil domains with high probabilities (see supplementary materials). Nevertheless, low coiled-coil forming probability has been observed in other members of the AT-2 such as YadA [87].

Based on the presence of the C-terminal region of YadA (PF03895), several hundred homologues related to invasins, immunoglobulin binding proteins, serum resistance proteins or haemagglutinins have been identified [87,88]. Using the translocation unit of identified members of the AT-2 family based on the presence of a YadA-like C-terminal domain (PF03895), phylogenetic analyses revealed that out of 18 deep-rooted branches, 10 display protein clustering (Fig. 6). Members of this family are only present in the domain *Bacteria*, in the phyla *Proteobacteria* (including the classes α-, β- and γ-*Proteobacteria*), *Cyanobacteria* and *Fusobacteria*. While the first characterised members of the AT-2 family, i.e., the YadA adhesin from *Yersinia enterocolitica*, the surface attachment antigen UspA2 from *Moraxella catarrhalis* [89], the immunoglobulin-binding proteins EibE from *E. coli* [90] and the keratinocyte-specific adhesin DsrA from *Haemophilus ducreyi* [91] are all found in cluster 1, all fusobacterial AT-2 displaying haemagglutinin and invasin repeats are found in cluster 10 (Fig. 6).

4. Discussion

A lack of genetic systems for the study of *F. nucleatum* has impeded the investigation of the molecular mechanisms

Table 6
AT-2 proteins of *F. nucleatum* subsp. *nucleatum* ATCC 25586

Locus name	Orthologue[a]	Length[b]	Signal sequence[c]	BetaWrap[d]	Annotation	Significant functional motif[e]
FN1499	FNV1729 (80%)	479	25 (YSA-AP)	119–222 (1.3×10^{-2})	Cell surface protein	Haemagglutinin repeats (PF05662); Invasin repeats (PF05658)
FN0471	–	348	30 (IEA-GT)	45–170 (1.3×10^{-3})	Cell surface protein	Haemagglutinin repeats (PF05662); Invasin repeats (PF05658)
FN0735	–	602	31 (IEA-GT)	294–406 (1.5×10^{-3})	Cell surface protein	Haemagglutinin repeats (PF05662); Invasin repeats (PF05658)

[a] Orthologues present in *F. nucleatum* subsp. *vincentii* ATCC 49256; percentage of similarity is indicated in parentheses whenever possible.
[b] Protein length expressed in number of amino acids including the signal peptide.
[c] Results from SignalP, PSORT and/or LipoP searches where the length of the signal peptide is given in number of amino acids and the cleavage site is given in parentheses.
[d] Position of the best wrap calculated by BetaWrap with the *P* value given in parentheses.
[e] Functional motifs were found using Pfam.

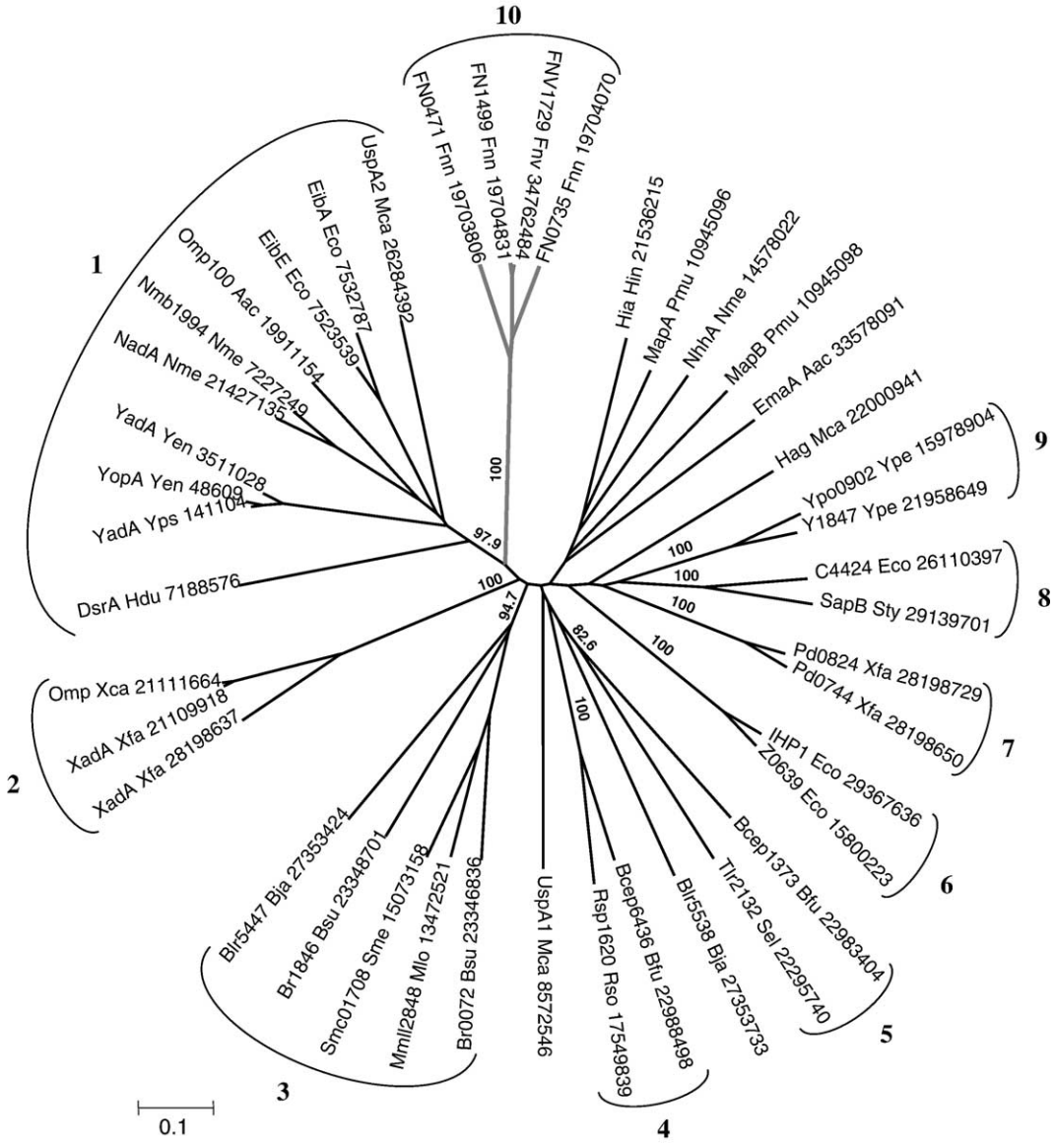

Fig. 6. Phylogenetic tree of currently recognised members of the Type Vc secretion family in relation to the newly found AT-2 proteins in *F. nucleatum* sp. *nucleatum* and *F. nucleatum* sp. *vincentii*. The tree is derived from analyses of the translocation unit of AT-2. The scale bar represents the evolutionary distance, i.e., the number of substitutions per site. The values on branches before nodes are the bootstrap confidence levels expressed in percentage. Branches containing fusobacterial protein(s) are greyed. Aac: *Actinobacillus actinomycetemcomitans*; Bfu: *Burkholderia fungorum*; Bja: *Bradyrhizobium japonicum*; Bsu: *Brucella suis*; Eco: *Escherichia coli*; Hdu: *Haemophilus ducrey*; Hin: *Haemophilus influenzae*; Mca: *Moraxella catarrhalis*; Mlo: *Mesorhizobium loti*; Nme: *Neisseria meningitidis*; Pmu: *Pasteurella multocida*; Rso: *Ralstonia solanacearum*; Sel: *Synechococcus elongatus*; Sme: *Sinorhizobium meliloti*; Sty: *Salmonella typhimurium*; Yen: *Yersinia enterocolitica*; Yps: *Yersinia pseudotuberculosis*; Xca: *Xanthomonas campestris*; Xfa: *Xylella fastidiosa*.

involved in virulence. In an effort to understand this organism at the metabolic and genetic level, and thereby shed light on its mode of pathogenesis, Kapatral et al. sequenced two strains of *F. nucleatum*. Comparative analyses of the sequenced strain *F. nucleatum* subsp. *nucleatum* ATCC 25586 and the draft genome of *F. nucleatum* subsp. *vincentii* ATCC 49256 suggested that the major protein secretion systems were missing [3,4]. However, as secreted proteins are the components by which bacteria interface with the external environment, and given what is known of the nature of *F. nucleatum*, it would appear to be intuitive that this organism would possess secreted proteins. Thus, reinvestigation of the *F. nucleatum* genomes was warranted. Here, we confirmed the Tat system (TC #2.A.64) was likely absent in *F. nucleatum* subsp. *nucleatum* and that inner membrane protein translocation seems to rely essentially on the Sec translocon. The chaperone/usher pathway (TC #1.B.11), as well as the Type I (ABC transporter: TC #3.A.1; MFP: TC #8.A.1; OMF: TC #1.B.17), Type III (TC #3.A.6) and Type IV (TC #3.A.7) protein secretion systems also seem to be missing in *F. nucleatum*. However, the complete secretion machinery for

a Tfp system (TC #3.A.15.2) and a complete range of proteins secreted through the Type V secretion system have been identified.

As co-aggregation bridge micro-organisms, fusobacteria are known to interact specifically with the inert tooth surface, mammalian cells and several bacterial species in the oral cavity to eventually form biofilms [92]. As *F. nucleatum* demonstrates this extraordinary adhesive ability, the discovery of a piliation system and the Type V secretion systems is of particular relevance to the biology of *F. nucleatum*. There is substantial evidence to support the notion that *F. nucleatum* possesses both lectin-like and proteinaceous adhesins, however, in general, these have not been identified at the genetic level [11,93,94]. Bacterial adhesion to a substratum is often characterised by initial adherence via pili followed by a more intimate association with the substratum through surface proteins [95]. Thus, the Tfp may provide the initial adherence required for *F. nucleatum* colonisation. Indeed, Tfp have been described in a variety of organisms including *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*, where they have been shown to bind to inert surfaces, bacterial cells and mammalian cells thereby mediating colonisation and biofilm formation [72,73]. The attachment of the Tfp pili to inert surfaces occurs via non-specific adhesion at the pilus tip, however, pili appear to bind to eukaryotic cells via specific interactions with carbohydrate receptors. In this respect, the Tfp may account for some of the lectin-like adherence patterns observed with *F. nucleatum*. Furthermore, in the absence of other notable pilus biogenesis loci, it seems that this Tfp locus could be responsible for the polarly localised pili observed by electron microscopy of *F. nucleatum* and which appeared to be instrumental in biofilm formation [96]. Notably, Tfp in other organisms are also polarly localised.

Tfp also mediate a kind of surface translocation, known as twitching motility, that depends on alternating extension and retraction of the pilus filament [72]. Notably, *F. nucleatum* possesses two nucleotide-binding proteins homologous to PilB and PilT, respectively. Although both Tfp and Type II protein secretion systems require PilB homologues to facilitate protein secretion, PilT homologues are required for pilus retraction and are unique to Tfp systems [72]. Taken together with the existence in an operonic structure of genes encoding PilA, PilC, PilN and PilQ homologues, as well as several prepilin-like proteins, it appears that *F. nucleatum* may be capable of assembling Tfp. Interestingly, the presence of *pilMNOP* genes has been demonstrated to be essential for twitching motility and pilus assembly in *P. aeruginosa* [72]. While a PilN homologue is apparent, the absence of obvious *pilM*, *pilO* and *pilP* counterparts may be due to mutational attrition of the *F. nucleatum* locus and suggests that the Tfp may be non-functional in this organism. However, a *pilP* homologue is lacking in *Synechocystis* sp., which possesses a functional Tfp system, indicating that PilP is not essential for every Tfp system (Beatson and Henderson, unpublished data). Furthermore, previous investigations suggested that *F. nucleatum* underwent 'gliding motility' though comparison of the *F. nucleatum* gliding motility with similar images of *P. aeruginosa* revealed this had identical features to twitching motility, and differed from the gliding motility associated with *Myxococcus xanthus* [97]. Thus, in light of the fact that *F. nucleatum* has previously been demonstrated to possess pili, and to undergo twitching-like motility, it seems likely that the genes located between *pilQ* and *pilD* are functional genes which are divergent from their proteobacterial counterparts but nevertheless might be sufficient for *F. nucleatum* Tfp assembly.

In addition to pili, members of the Type V secretion system, such as the *E. coli* autotransporter Antigen 43 and the *Bordetella bronchiseptica* TpsA protein filamentous haemagglutinin, have been demonstrated to play a role in biofilm formation. In the case of Antigen 43, biofilm formation is known to be due to the ability of Antigen 43 to promote cell–cell interactions [98]. In a similar fashion, a number of the Type Vc proteins such as the *Yersina* YadA, *E. coli* Ssa and the Vomp proteins from *Bartonella quintana*, promote autoaggregation of bacterial cells [99–101]. Thus, as *F. nucleatum* possesses the ability to interact via bacterial cell–cell contact, and to autoagglutinate, any number of the Type V secreted proteins may contribute to cell–cell interaction and thus biofilm formation.

Several studies have demonstrated that *F. nucleatum* adheres to extracellular matrix components and mammalian cells prior to invasion of gingival cells [11]. There is ample evidence from a variety of different bacteria to support a role for autotransporters, TpsA proteins and members of the AT-2 subfamily in adhesion to both extracellular matrix components and cell membrane constituents, as well as invasion of mammalian cells [91,102–106]. Interestingly, invasin/intimin cell adhesion and RGD cell-attachment motifs are found in the passenger domains of several putative fusobacterial Type V secreted proteins. In enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC), diarrheal disease is caused by disruption of the intestinal epithelium through intimate attachment of the bacteria to enterocytes, a process mediated by intimin, an outer membrane protein that is homologous to the invasins of pathogenic *Yersinia* [107]. Furthermore, RGD motifs have been shown to mediate adhesion to mammalian cells through interaction with β1 integrins [108], and indeed, this has been proven for the *B. pertussis* Type Vb protein, filamentous haemagglutinin [109]. While it can be argued that the probability of RGD motifs occurring without evidence of functionality is high, in autotransporters this motif is very often associated with proteins predicted to have cell adhesion properties [30]. It is perhaps worth noting that several previously described Type V secreted adhesins have been demonstrated to elicit IL-8, a chemokine involved in inducing an inflammatory response which is a key step in progress to periodontal disease [110]. Thus, in the absence

of any other defined protein secretion pathways, aside from the Tfp system, it appears that the Type V secretion pathway may be involved in some fashion with the ability of *F. nucleatum* to adhere to eukaryotic moieties and to invade host cells, and thereby these systems could contribute to the pathogenesis of this organism.

In *F. nucleatum*, most proteins of the Type Vb are associated to the secretion of putative hemolysins. In *Serratia marcescens*, where the function of such hemolysins/cytolysins has been undoubtedly the most investigated, ShlA contributes to colonisation of infected tissue by inducing cytotoxic effects and final lysis of infected eukaryotic cells, namely erythrocytes but also fibroblasts and epithelial cells [111]. While it remains possible that the TpsA proteins may play a role in cytotoxicity similar to that observed for ShlA, the induction of cytotoxic effects has not previously been described for *F. nucleatum*. It is worth mentioning that the presence of RGD motifs, such as those already noted for a number of the Type V secreted proteins, has been implicated in the ability of certain proteins to induce apoptosis and that the presence of these proteins may explain how *F. nucleatum* induces apoptosis in polymorphonuclear cells [112,113].

The pathophysiology of periodontitis is a complex process involving both bacterial and host proteolytic enzymes in the destruction of the gingival tissue. Previous investigations have identified proteases elaborated by *F. nucleatum* but which have not been characterised at the genetic level. In the current investigation, a cysteine protease autotransporter has been identified in *F. nucleatum* subsp. *nucleatum*; to our knowledge, this constitutes the first report of an autotransporter protein bearing such a catalytic site. The passenger domain of this putative autotransporter also harbors filamin and spectrin repeats, suggesting possible interactions with cytoskeletal proteins. Interestingly, in the oral pathogenic bacterium *Porphyromonas gingivalis*, cysteine proteases, termed gingipains, are involved in fibrinogen/fibrin degradation, induction of vascular permeability and activation of the blood coagulation system leading to gingival crevicular fluid production, progression of inflammation and subsequent alveolar bone loss in periodontitis [114,115]. In addition, gingipains degrade macrophage CD14, inhibiting activation of the leukocytes, and thereby facilitating persistent colonisation of *P. gingivalis*. In light of its prolonged colonisation and contribution to periodontal disease, one can hypothesise a similar role for the *F. nucleatum* cysteine protease autotransporter.

In addition to the cysteine protease described above, two serine protease autotransporters of the subtilase family were identified in *F. nucleatum* during this study. Despite the extensive investigation of the secretion of the subtilisin-like serine protease family of autotransporters [116], there is little information about the role of these proteins in vivo. Nevertheless, the *Bordetella pertussis* and *Neisseria meningitidis* subtilisin autotransporters, SphB1 and NalP, are clearly involved in auto- and trans-maturation of bacterial proteins, including TpsA exoproteins, e.g., filamentous haemagglutinin [29,117]. As *F. nucleatum* clearly possesses several TpsA proteins, it can be envisaged that these subtilisins also perform a similar role in TpsA maturation. However, a recent investigation identified, at the biochemical level, a serine protease capable of degrading extracellular matrix proteins such as fibronection, fibrinogen and collagens [118]. The degradation of extracellular matrix proteins by oral bacteria is a significant contributor to invasion of the gingival tissue and subsequent damage of periodontal tissues. It is possible that this serine protease is one of the autotransporters identified in this study. Notably, like the Neisserial IgA1 protease autotransporters, this enzyme was also capable of degrading IgA, which may help *F. nucleatum* in evading the host immune system.

From the prolonged colonisation of the periodontal region with *F. nucleatum*, one might speculate that this organism possesses the ability to avoid the host immune response [13]. Indeed, several autotransporters and Type Vc secreted proteins have been implicated in the degradation of antibacterial serum components, and in providing serum resistance, indicating that the homologous proteins may provide a similar function in *F. nucleatum*. Interestingly, inspection of the genes encoding TpsA and/or TpsB proteins at the nucleotide level suggested that these proteins are subject to phase variation by translational slipped-strand mispairing, thus allowing expression of these genes to be switched on and off at appropriate times. Indeed, phase variation has been described for a number of genes encoding proteins of the Type V secretion system [119,120] and notably slipped-strand mispairing regulates expression of the HMW1a and HMW2a TpsA proteins of *H. influenzae*. It has long been proposed that phase variation is a mechanism for immune evasion by pathogenic organisms, as surface components, such as the large TpsA proteins, are likely to be highly immunogenic and therefore prime targets for protective immune responses.

Besides the TpsA/B homologues, an Omp85-like protein is present in *F. nucleatum* subsp. *nucleatum*. It is worth stressing that despite being related to the TpsB family [80], it has never been demonstrated that Omp85-like proteins are involved in the translocation and secretion of proteins through outer membranes. However, following Omp85 depletion, which is an essential protein for cell viability, it has been shown that unassembled forms of various outer membrane proteins, including TpsB and autotransporter proteins, accumulated [121]. However, controversy exists over a direct or an indirect involvement of Omp85 in outer membrane protein assembly [122–124]. Nevertheless, from an evolutionary point of view, the presence of Omp85-like protein in chloroplasts, the phyla *Proteobacteria*, *Bacteroidetes*, *Aquificae*, *Chlamydiae*, *Spirochaetes*, *Cyanobacteria* and in *F. nucleatum*, which belongs to a phylum at the junction between Gram-negative and Gram-positive bacteria, suggests Omp85 is an ancient protein linked to the appearance of outer membrane [80,121,123].

The present genomic analysis of the protein secretion systems in *F. nucleatum* has also pointed out that for some reason a large number of genes related to the Type V secretion system were misannotated at their start codon and/or frameshifted. As already mentioned, translational slipped-strand mispairing could explain frame shift in some cases. However, it appears that the misannotated start codons may have arisen through the use of automated annotation software and thus similar studies should be approached with an air of caution. In addition, it is clear from analyses of the *F. nucleatum* subsp. *vincentii* genome that many essential genes possess frameshifts. These observations suggest it is probably premature to make any qualitative judgements from the comparison of *F. nucleatum* subsp. *vincentii* draft genome with *F. nucleatum* subsp. *nucleatum* [125].

In conclusion, using *Chlamydiae* as an example, where the polymorphic membrane proteins were first identified as autotransporters by bioinformatic analyses and later biochemically characterised [27,30,81], this genomic analysis of protein secretion should promote future in vivo investigations of the Type V secretion system in *F. nucleatum*. Further work is necessary to determine whether or not these putative proteins are expressed, secreted and functional, as well as to establish their roles in bacterial pathogenicity. Moreover, biochemical characterisation of these systems might have implications for vaccine development and treatment of *F. nucleatum*, namely in periodental diseases.

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