Phylogenetic comparisons between Type IX Secretion System (T9SS) protein components suggest evidence of horizontal gene transfers

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Porphyromonas gingivalis is one of the major bacteria that causes periodontitis. Chronic periodontitis is a severe form of periodontal disease that ultimately lead to tooth loss. Virulence factors that contribute to periodontitis are secreted by Type IX Secretion System (T9SS). There are aspects of T9SS protein components that have yet to be characterised. Thus, the aim of this study is to investigate phylogenetic relationship between members of 20 T9SS component protein families. The Bayesian Inference (BI) trees for 19 T9SS protein components exhibit monophyletic clades for all major classes under Bacteroidetes with a strong support for the monophyletic clades or its subclades that is consistent with phylogeny exhibited by the constructed BI tree of 16S rRNA. The BI tree of PorR is different from the 19 BI trees of T9SS protein components as it does not exhibit monophyletic clades for all major classes under Bacteroidetes. There is a strong support for the phylogeny exhibited by BI tree of PorR deviates from the phylogeny based on 16S rRNA. Hence, it is possible that *porR* gene is subjected to horizontal transfer as it is known that virulence factor gene could be horizontally transferred. Seven genes (*porR* included) that involved in the biosynthesis of A-LPS are found to be flanked by insertion sequences (IS5 family transposons). Therefore, the intervening DNA segment that contains *porR* gene might be transposed and subjected to conjugative transfer. Thus, the seven genes can be co-transferred via horizontal gene transfer. The BI tree of UgdA does not exhibit monophyletic clades for all major classes under Bacteroidetes which is similar to the BI tree of PorR (both are a part of the seven genes). Both BI trees also exhibit similar topology as the four identified clusters with a strong support have similar relative positions to each other in both BI trees. This reinforces the possibility that *porR* and the other six genes might be horizontally transferred. Other than the BI tree of PorR, the other 19 BI trees of T9SS protein components also exhibit evidence of horizontal gene transfer. However their genes might undergo horizontal gene transfer less frequently compared to *porR* because the intervening DNA segment that contains *porR* is easily exchanged

between bacteria under Bacteroidetes due to the presence of insertion sequences (IS5 family transposons) that flanking it. In conclusion, this study can provide a better understanding about the phylogeny of T9SS protein components.

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Abstract

Porphyromonas gingivalis is one of the major bacteria that causes periodontitis. Chronic periodontitis is a severe form of periodontal disease that ultimately lead to tooth loss. Virulence factors that contribute to periodontitis are secreted by Type IX Secretion System (T9SS). There are aspects of T9SS protein components that have yet to be characterised. Thus, the aim of this study is to investigate phylogenetic relationship between members of 20 T9SS component protein families. The Bayesian Inference (BI) trees for 19 T9SS protein components exhibit monophyletic clades for all major classes under Bacteroidetes with a strong support for the monophyletic clades or its subclades that is consistent with phylogeny exhibited by the constructed BI tree of 16S rRNA. The BI tree of PorR is different from the 19 BI trees of T9SS protein components as it does not exhibit monophyletic clades for all major classes under Bacteroidetes. There is a strong support for the phylogeny exhibited by BI tree of PorR deviates from the phylogeny based on 16S rRNA. Hence, it is possible that *porR* gene is subjected to horizontal transfer as it is known that virulence factor gene could be horizontally transferred. Seven genes (*porR* included) that involved in the biosynthesis of A-LPS are found to be flanked by insertion sequences (IS5 family transposons). Therefore, the intervening DNA segment that contains *porR* gene might be transposed and subjected to conjugative transfer. Thus, the seven genes can be co-transferred via horizontal gene transfer. The BI tree of UgdA does not exhibit monophyletic clades for all major classes under Bacteroidetes which is similar to the BI tree of PorR (both are a part of the seven genes). Both BI trees also exhibit similar topology as the four identified clusters with a strong support have similar relative positions to each other in both BI trees. This reinforces the possibility that *porR* and the other six genes might be horizontally transferred. Other than the BI tree of PorR, the other 19 BI trees of T9SS protein components also exhibit evidence of horizontal gene transfer. However their genes might undergo horizontal gene transfer less frequently compared to *porR* because the intervening DNA segment that contains *porR* is easily exchanged between bacteria under Bacteroidetes due to the presence of insertion sequences (IS5 family transposons) that flanking it. In conclusion, this study can provide a better understanding about the phylogeny of T9SS protein components.

Introduction

Periodontitis is a form of periodontal disease that is driven by the inflammatory conditions that have deteriorating effects on the structures that support the teeth that include gingiva (gum), alveolar bone, and periodontal ligament. Prolonged inflammatory conditions in chronic periodontitis can cause the destruction of those supporting structures that ultimately lead to tooth loss and might contribute to the systemic inflammation (Kinane, Stathopoulou & Papapanou, 2017; Escobar et al., 2018). This is evidenced by its implications in systemic diseases such as atherosclerosis (Gotsman et al., 2007), aspiration pneumonia (Benedyk et al., 2016), cancer (Gao et al., 2016), rheumatoid arthritis (Laugisch et al., 2016), and diabetes mellitus (Khader et al., 2006). *Porphyromonas gingivalis* is an oral pathogen that is frequently associated with periodontitis and it is found to acquire Type IX Secretion System (T9SS); a bacterial secretion system that is unique to gram-negative bacteria under Bacteroidetes phylum (Sato et al., 2010).

T9SS exhibits diverse roles among species of bacteria under Bacteroidetes. Other than transporting virulence factors such as gingipains and peptidylarginine deiminase in *P. gingivalis* that can cause human oral diseases (Potempa, Pike & Travis, 1995; Maresz et al., 2013), T9SS also transporting virulence factors such as chondroitin sulfate lyases that can cause columnaris disease which is a form of fish disease. *Flavobacterium columnare*, a fish pathogen that contributes to the epidemic that occurred among wild and cultured fishes, is found to acquire T9SS. This epidemic poses a problem to the aquaculture industry as columnaris disease can significantly increase the mortality rate among cultured fishes and thus threatened the industry output (Li et al., 2017). T9SS also involved in the transport of non-virulence factors such as cargo proteins that formed the bacterial gliding motility apparatus in *Flavobacterium johnsoniae* that aids in its motility (Nakane et al., 2013) and enzymes that are importance for lignocellulose digestion in the rumen of ruminants that become the hosts for the *Candidatus Paraporphyromonas polyenzymogenes* (Naas et al., 2018).

Gram-negative bacteria have an outer membrane (OM) that acts as an impermeable layer that prevents the free movement of hydrophilic and hydrophobic molecules across it. This is because of the presence of lipopolysaccharides (LPS) within the outer leaflet of OM. Outer membrane protein that is embedded in the OM usually forms a channel to allow small molecules to pass through it (Nikaido, 2003; Hong et al., 2006). However, large molecules such as proteins require larger channel to pass through the OM. Hence, secretion systems are developed by bacteria to enable coordinated transport of specific cargo proteins across the OM. Currently, there are nine different types of secretion system evolved by bacteria. T9SS is restricted to bacteria under Bacteroidetes (Sato et al., 2010; Lasica et al., 2017).

T9SS is consisted of many different protein components that perform coordinated roles to ensure proper translocation and modification of its cargo proteins. These roles can be categorised into four major functions: translocation, modification, energetic, and regulation (Sato et al., 2010; Lasica et al., 2017; Naito et al., 2019). Initially, the cargo proteins of T9SS are translocated across the inner membrane (IM) via Sec translocon where the signal peptide (SP) of

cargo proteins is cleaved (Rahman et al., 2003). The cargo proteins also acquired C-terminal domain (CTD) that interacts with the $PorK_2L_3M_2N_2$ trans-envelope complex to translocate cargo proteins across periplasm (Vincent et al., 2017; Vincent, Chabalier & Cascales, 2018) (Fig. 1). PorE has been suggested to form the scaffold of periplasm complex that translocates cargo proteins across the periplasm (Heath et al., 2016; Naito et al., 2019). SprA (ortholog of Sov in *F. johnsoniae*) has been proposed as the secretion pore that translocates cargo proteins across OM (Lauber et al., 2018). PorV acts as an outer membrane shuttle protein that delivers the cargo proteins to the attachment complex (Glew et al., 2017) (Fig. 1). In the attachment complex, PorU cleaves the CTD of cargo protein. Then, it is glycosylated with anionic lipopolysaccharide (A-LPS) delivered by PorZ at the cleaved site (Glew et al., 2012, 2017). After both post-translational modifications, the cargo protein will be anchored to cell surface by A-LPS (Lasica et al., 2016; Glew et al., 2017) (Fig. 1). PorX and PorY formed a two-component system (TCS) that regulates the operon of *por* genes (*porP*, *porK*, *porL*, *porM*, and *porN*) via SigP (Vincent et al., 2017; Kadowaki et al., 2016) (Fig. 1). PorR is an aminotransferase that involves in the Wbp pathway that biosynthesis the structural repeating unit of anionic polysaccharide (APS) (Shoji et al., 2002; Shoji et al., 2014) (Fig. 1). Despite that, there are T9SS components without known functions (PorP, PorT, PorW, Omp17, PorF, and PorG) (Fig. 1) and a few aspects of T9SS components that have yet to be characterised (Nguyen et al., 2009; Saiki & Konishi, 2010; Sato et al., 2010; Gorasia et al., 2016; Naito et al., 2019; Taguchi et al., 2016).

This work aims to characterise the phylogeny of T9SS protein components. Phylogenetic analysis was performed on the members of 20 T9SS component protein families that have been reported (Emrizal & Muhammad, 2018). The Bayesian Inference (BI) trees for 19 T9SS protein components exhibit monophyletic clades for all major classes under Bacteroidetes with a strong support for the monophyletic clades or its subclades that is consistent with phylogeny exhibited by the constructed BI tree of 16S rRNA. The BI tree of PorR is different from the other 19 BI trees as it does not exhibit monophyletic clades for all major classes under Bacteroidetes. There is also a strong support for the phylogeny exhibited by BI tree of PorR. Thus, there is a possibility that *porR* gene is subjected to horizontal transfer as it is known that virulence factor gene could be horizontally transferred (Hirt, Schlievert & Dunny, 2002). Seven genes including *porR* that involved in the biosynthesis of A-LPS are found to be flanked by insertion sequences (IS5 family transposons). This suggests that the intervening DNA segment that contains *porR* can be transposed and subjected to conjugative transfer (Thomas & Nielsen, 2005; Brochet et al., 2009). Thus, the seven genes might be co-transferred via horizontal gene transfer. The BI trees of PorR and UgdA (both are a part of the seven genes) exhibit similarities. This reinforces the possibility that *porR* and the other six genes might undergo horizontal gene transfer. Other than the BI tree of PorR, the BI trees of other 19 components also exhibit evidence of horizontal gene transfer. However, for the genes that encode those 19 components, they might undergo horizontal gene transfer less frequently compared to *porR* because the intervening DNA segment that contains *porR* is easily exchanged between bacteria under Bacteroidetes due to the presence of IS5 family transposons that flanking it.

Materials & Methods

Construction of multiple sequence alignments of T9SS protein components

The multiple sequence alignments for each T9SS protein component were built using the putative members of T9SS component protein families. The pipeline that was used to select those members has been reported (Emrizal & Muhammad, 2018). The pipeline was used to filter out false positives among the homologs that have been identified through homology searching using BLASTP which was performed using T9SS component protein sequences retrieved from NCBI protein database that were searched against a local BLAST database constructed from completely sequenced bacterial proteomes from Genbank. The selection criteria used in the pipeline: e-value ≤ 0.001, query coverage > 60%, and Bacteroidetes homolog with the lowest e-value for bacterial strains with multiple hits; can minimise the possibility of false positives inclusion (Emrizal & Muhammad, 2018). The sequences of protein homologs used to build the multiple sequence alignments for each T9SS component were provided in FASTA format as a Supplemental Information (Data S1).

The multiple sequence alignments were constructed using MAFFT version (7.402) (Katoh et al., 2002) on the CIPRES computing cluster (Miller, Pfeiffer & Schwartz, 2010). The constructed multiple sequence alignments are in FASTA format. Unreliable alignment regions in the multiple sequence alignments were assessed using Guidance 2 version (2.02) (Sela et al., 2015) on the CIPRES computing cluster (Miller, Pfeiffer & Schwartz, 2010). Columns with low confidence were removed from the multiple sequence alignments. The format of multiple sequence alignments was converted into relaxed interleaved PHYLIP format using online Format Converter (https://www.hiv.lanl.gov/content/sequence/FORMAT_CONVERSION/form.html). The multiple sequence alignments in relaxed interleaved PHYLIP format were manually edited into NEXUS format.

Determination of amino acid substitution models for multiple sequence alignments of T9SS protein components

The multiple sequence alignments in relaxed interleaved PHYLIP format (Data S2) were used by ProtTest version (3.4.2) (Guindon & Gascuel, 2003; Darriba et al., 2011) to determine amino acid substitution model to be used for each alignment in the phylogenetic analysis. Graphical user interface (GUI) version of ProtTest was used to test each alignment against 10 amino acid substitution model matrices (Blosum62, CpREV, Dayhoff, JTT, MtMam, MtREV, RtREV, VT, WAG, and LG) with any combination of among-site rate variation (no rate variation across sites, gamma-shaped rate variation across sites (+G), a proportion of invariable sites (+I), or gamma-shaped rate variation across sites with a proportion of invariable sites (+G+I)) and stationary amino acid frequencies (Dirichlet or fixed (empirical) (+F)). The best model according to Bayesian Information Criterion (BIC) (Schwarz, 1978) was selected to be used in the phylogenetic analysis for that alignment.

Bayesian Inference (BI) analysis for multiple sequence alignments of T9SS protein components

Bayesian Inference (BI) analysis was performed using multiple sequence alignments in NEXUS format (Data S3). The BI analysis was performed using MrBayes version (3.2.6) (Huelsenbeck & Ronquist, 2001) on the CIPRES computing cluster (Miller, Pfeiffer & Schwartz, 2010) for alignments of 14 components (PorK, PorL, PorM, PorN, PorP, PorQ, PorT, PorU, PorV, SigP, Omp17, PorE, PorF, and PorG). The BI analysis for each alignment was performed with the selected amino acid substitution model and two independent runs, for 50,000,000 generations, each with four chains, with a sampling frequency of every 5,000, and a burn-in of 25%. Beagle CPU was utilised to speed up the BI analysis.

The BI analysis for the other 6 components (PorR, Sov, PorW, PorX, PorY, and PorZ) was performed using command-line MrBayes version (3.2.6) (Huelsenbeck & Ronquist, 2001) on a desktop with Nvidia Titan V GPU and CUDA driver version (10.1) installed. The BI analysis for each alignment was performed with the selected amino acid substitution model and two independent runs, for 50,000,000 generations, each with four chains (PorR, Sov, PorW) or eight chains (PorX, PorY, and PorZ), with a sampling frequency of every 5,000, and a burn-in of 25%. Beagle GPU was utilised to speed up the BI analysis. The constructed BI trees were visualised and annotated using online iTOL version (4.4.2) (Letunic & Bork, 2019).

Construction of Bayesian Inference (BI) tree of 16S ribosomal RNA (rRNA)

The 16S ribosomal RNA (rRNA) sequences have been used to construct the current universal tree of life (Winker & Woese, 1991; Pylro et al., 2012). Thus, the BI tree of 16S rRNA has been constructed in this work to compare it with the BI trees of T9SS protein components. A pre-formatted BLAST database of microbial 16S rRNA sequences was retrieved from NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db/). The 16S rRNA sequence from *Porphyromonas gingivalis* ATCC 33277 (NR_040838.1) was retrieved from NCBI (https://www.ncbi.nlm.nih.gov/gene) and it was searched against that database using local BLASTN (Altschul et al., 1990). The pipeline mentioned above was used to select homologs of 16S rRNA gene in species under Bacteroidetes that are also found to acquire homologs of T9SS protein components in this work. For those species that their 16S rRNA sequences could not be retrieved from the microbial 16S rRNA BLAST database, their 16S rRNA sequences were retrieved directly from either the NCBI Gene (https://www.ncbi.nlm.nih.gov/gene) or NCBI Nucleotide (https://www.ncbi.nlm.nih.gov/nuccore/). The selected 16S rRNA sequences were provided in FASTA format as a Supplemental Information (Data S1).

The sequences were used to build the multiple sequence alignment of 16S rRNA using MAFFT version (7.402) (Katoh et al., 2002) and unreliable alignment regions in the multiple sequence alignment were assessed using Guidance 2 version (2.02) (Sela et al., 2015) on the CIPRES computing cluster (Miller, Pfeiffer & Schwartz, 2010). Columns with low confidence were removed from the multiple sequence alignment. The alignment in FASTA format (Data S2) was used to determine the best nucleotide substitution model to be used in the phylogenetic

analysis. Graphical user interface (GUI) version of ModelTest (Darriba et al., 2019) was used to test the alignment against 3 nucleotide substitution model matrices (GTR, HKY85, and F81) with any combination of among-site rate variation (no rate variation across sites, gamma-shaped rate variation across sites (+G), a proportion of invariable sites (+I), or gamma-shaped rate variation across sites with a proportion of invariable sites (+G+I)) and stationary amino acid frequencies (Dirichlet or fixed (empirical) (+F)). The best model according to Bayesian Information Criterion (BIC) (Schwarz, 1978) was selected to be used in the phylogenetic analysis for that alignment.

BI analysis was performed using the alignment in NEXUS format (Data S3). The analysis was performed using MrBayes version (3.2.6) (Huelsenbeck & Ronquist, 2001) on the CIPRES computing cluster (Miller, Pfeiffer & Schwartz, 2010) with the selected nucleotide substitution model and two independent runs, for 50,000,000 generations, each with four chains, with a sampling frequency of every 5,000, and a burn-in of 25%. Beagle CPU was utilised to speed up the BI analysis. The BI tree of 16S rRNA was visualised and annotated using online iTOL version (4.4.2) (Letunic & Bork, 2019).

Identification of *porR* and its neighbouring genes arrangement in *Porphyromonas gingivalis* ATCC 33277 genome

The sequence of *P. gingivalis* ATCC 33277 genome and annotation files of the genome were retrieved from Genbank (Naito et al., 2008). The *P. gingivalis* ATCC 33277 genome sequence and its annotation files were provided in the Supplemental Information (Data S4). The part of *P. gingivalis* ATCC 33277 genome sequence that contains the *porR* and its neighbouring genes was extracted. Then, it was searched against the non-redundant protein sequences (nr) database using online BLASTX. The search was narrowed down to the proteome of *P. gingivalis* ATCC 33277 only. The maximum target sequences were set at highest value available which is 20,000. Other parameters were left at its default values (Altschul et al., 1990). Only the matches with 100% percentage identity and 0 e-value were used to annotate the part of *P. gingivalis* ATCC 33277 genome sequence that contains *porR* gene.

Construction of Bayesian Inference (BI) tree of UgdA

Based on identification of *porR* neighbouring genes, the two genes that involve in Wbp pathway (*ugdA* and *porR*) are found to be within the intervening DNA segment that is flanked by IS5 family transposons. Thus, BI tree of UgdA was constructed to be compared with BI tree of PorR. The pipeline mentioned above was used to select homologs of UgdA (Data S1) and construct the multiple sequence alignment of UgdA with low confidence columns being removed. The alignment in relaxed interleaved PHYLIP format (Data S2) was used to determine the best amino acid substitution model. BI analysis was performed using UgdA alignment in NEXUS format (Data S3). The analysis was performed using command-line MrBayes version (3.2.6) (Huelsenbeck & Ronquist, 2001) on a desktop with Nvidia Titan V GPU and CUDA driver version (10.1) installed with the selected amino acid substitution model and two independent

runs, for 50,000,000 generations, each with four chains, with a sampling frequency of every 5,000, and a burn-in of 25%. Beagle GPU was utilised to speed up the BI analysis. The constructed BI tree was visualised and annotated using online iTOL version (4.4.2) (Letunic & Bork, 2019).

Results

Bayesian Inference (BI) trees of T9SS protein components

Bayesian Inference (BI) trees are constructed from the multiple sequence alignments of putative members of T9SS component protein families that have been reported (Emrizal & Muhammad, 2018). The characteristics of alignments and the best amino acid substitution model that has been selected for each alignment are shown in Table 1. The selected amino acid substitution model for each alignment defines the parameters that are used for BI analysis for each alignment. The unrooted BI trees of T9SS protein components are shown (Figs. 2-6). The identified monophyletic clades that formed by terminal nodes that belong to the same class under Bacteroidetes are denoted by solid curves (Figs. 2-6). The monophyletic clades or its subclades with a strong support (posterior probability value > 0.95) are denoted by dashed curves (Figs. 2-6).

Out of 20 BI trees of T9SS protein components, only 19 exhibit monophyletic clades for all major classes under Bacteroidetes (Figs. 2-6). Major classes are those with more than five families under the classes (Bacteroidia, Cytophagia, and Flavobacteriia) while minor classes are those with less than or equal to five families under the classes (Chitinophagia, Sphingobacteriia, Saprospiria, Incertae sedis, and unclassified). Nine of the BI trees (PorK, Sov, PorT, PorV, PorW, PorX, Omp17, PorE, and PorF) exhibit monophyletic clades for all major classes under Bacteroidetes with a strong support. Ten of the BI trees (PorL, PorM, PorN, PorP, PorQ, PorU, PorY, PorZ, SigP, and PorG) exhibit strong support for the monophyletic clades or its subclades for all major classes under Bacteroidetes (Figs. 2-6). Despite the presence of PorR homologs from species under Bacteroidia, Cytophagia, and Flavobacteriia, the BI tree of PorR does not exhibit monophyletic clades for all major classes under Bacteroidetes (Fig. 3C). Thus, the BI tree of PorR is different compared to the other 19 BI trees of T9SS protein components that exhibit monophyletic clades for all major classes under Bacteroidetes.

Some of the terminal nodes of 19 BI trees of T9SS protein components are out of its expected monophyletic clades (Figs. 2-6). The species corresponding to those terminal nodes are listed in Table S1. There are species that frequently have their terminal nodes out of its expected monophyletic clades such as *Fluviicola taffensis* DSM 16823, bacterium L21-Spi-D4, *Owenweeksia hongkongensis* DSM 17368, and *Draconibacterium orientale*. The terminal nodes corresponding to *F. taffensis* DSM 16823 are found to be out of its expected monophyletic clades in 14 out of 19 BI trees (except PorK, PorN, PorP, PorU, and SigP). The terminal nodes corresponding to bacterium L21-Spi-D4 are found to be out of its monophyletic clades in 10 out of 19 BI trees (except PorK, PorL, PorM, Sov, PorT, PorU, PorX, PorY, and PorE). The terminal

nodes corresponding to *O. hongkongensis* DSM 17368 are found to be out of its expected monophyletic clades in 6 out of 19 BI trees (PorM, PorP, PorV, PorY, Omp17, and PorE). The terminal nodes corresponding to *D. orientale* are found to be out of its expected monophyletic clades in 7 out of 19 BI trees (PorN, PorP, PorV, PorW, PorY, SigP, and Omp 17). The 20 BI trees with terminal nodes labelled with its corresponding species and support values for each branch are shown are provided in the Supplemental Information (Figs. S1-S20).

Bayesian Inference (BI) tree of 16S rRNA

The BI tree of 16S rRNA is constructed from the multiple sequence alignment of 16S rRNA homologs from species that are identified to also acquire T9SS protein homologs. Out of 181 species that acquire T9SS protein homologs, only 16S rRNA sequences from 144 species are able to be retrieved from NCBI. The characteristic of 16S rRNA alignment and the best nucleotide substitution model that has been selected for that alignment are shown in Table 1. The unrooted BI tree of 16S rRNA is shown in Fig. 7. The identified monophyletic clades that formed by terminal nodes that belong to the same class under Bacteroidetes are denoted by solid curves (Fig. 7). The monophyletic clades or its subclades with a strong support (posterior probability value > 0.95) are denoted by dashed curves (Fig. 7).

The BI tree of 16S rRNA is constructed to be compared to the BI trees of T9SS protein components. The 16S rRNA exhibits monophyletic clades for all major classes under Bacteroidetes with a strong support (Fig. 7) similar to the 19 BI trees of T9SS protein components. The 16S rRNA also exhibits monophyletic clades for all minor classes under Bacteroidetes with a strong support denoted by 4 monophyletic clades of red, pink, yellow and orange circles (Fig. 7). None of the 20 BI trees of T9SS protein components exhibit phylogeny of the minor classes that is consistent to the phylogeny exhibits by the 16S rRNA tree (Fig. 2-7). Hence, minor classes are excluded in the comparison between 20 BI trees of T9SS protein components. The BI tree of 16S rRNA with terminal nodes labelled with its corresponding species and support values for each branch are shown is provided in the Supplemental Information (Fig. S21).

Arrangement of *porR* and its neighbouring genes in *P. gingivalis* ATCC 33277 genome

As shown in Fig. 8, *porR* and its neighbouring genes are flanked by IS5 family transposons. The IS5 family transposon (cyan rectangles) encodes IS5 family transposase that cleaves the flanking 12 bp inverted repeats (purple triangles) (Fig. 8). This might suggest the possibility that intervening DNA segment that contains seven genes that involved in A-LPS biosynthesis (yellow rectangles) can undergo transposition and possibly subjected to conjugative transfer (Fig. 8) (Thomas & Nielsen, 2005; Brochet et al., 2009). *porR* (PGN_1236) and *ugdA* (PGN_1243) genes (Fig. 8) have been reported to be involved in Wbp pathway that is important for the biosynthesis of structural sugar (di-acetylated glucuronic acid) of A-LPS (Shoji et al., 2002; Shoji et al., 2014). *porS* (PGN_1235) and *wzy* (PGN_1242) genes (Fig. 8) have been reported to participate in the assembly of A-LPS in bacterial inner membrane (Shoji et al., 2013). *gtfB*

(PGN_1251) and *gtfE* (PGN_1240) glycosyltransferases genes (Fig. 8) are important for A-LPS biosynthesis while *rfa* (PGN_1255) glycosyltransferase gene (Fig. 8) is important for biosynthesis of lipid A-core portion of A-LPS (Shoji et al., 2018).

Bayesian Inference (BI) tree of UgdA

The BI tree of UgdA is constructed from the multiple sequence alignment of UgdA homologs that are identified using the same pipeline that has been reported to select T9SS protein homologs (Emrizal & Muhammad, 2018). The characteristic of UgdA alignment and the best amino acid substitution model that has been selected for that alignment are shown in Table 1. The unrooted BI tree of UgdA is shown in Fig. 9B. The unrooted BI tree of PorR is also shown in Fig. 9A to be compared with the BI tree of UgdA. Both BI trees do not exhibit monophyletic clades for all major classes under Bacteroidetes. Both BI trees also exhibit similar topology. Four similar clusters (I, II, III, and IV) have been identified between both BI trees. Cluster I consists primarily of terminal nodes from Flavobacteriia and a few terminal nodes from other classes. Cluster II consists of terminal nodes from Porphyromonas, Tannerella, and Parabacteroides genera. Cluster III consists of terminal nodes from Rufibacter and Hymenobacter genera. Cluster IV consists of terminal nodes from Prevotella, Bacteroides, Proteiniphilum and other genera. The BI tree of UgdA with terminal nodes labelled with its corresponding species and support values for each branch are shown is provided in the Supplemental Information (Fig. S22).

Taxonomic distribution of T9SS protein components

As shown in the 20 BI trees of T9SS components (Figs. 2-6), only bacteria under Bacteroidia, Flavobacteriia, and Chitinophagia classes acquired the 20 components investigated in this work. The bacteria under Cytophagia class acquired only 19 protein components (except PorN). The bacteria under Saprospiria class acquired only 18 protein components (except PorL and PorG). The bacteria under Sphingobacteriia class acquired only 17 protein components (except PorQ, PorU, and PorZ). The bacteria under unclassified acquired only 17 protein components (except PorN, PorU, and PorG). The bacteria under Incertae sedis class acquired only 11 protein components (PorQ, PorR, Sov, PorU, PorV, PorX, PorY, PorZ, SigP, Omp17, and PorF).

The finding in this work is consistent with the taxonomic distribution of T9SS components among bacteria under Bacteroidetes where it has been reported that Bacteroidia, Flavobacteriia, Cytophagia, Sphingobacteriia, and Incertae sedis classes acquired T9SS component homologs (McBride & Zhu, 2013). However, comparing the reported taxonomic distribution of T9SS components to the finding in this work, we have identified other species under Chitinophagia, Saprospiria, and unclassified that acquired T9SS component homologs. Those species and T9SS component homologs they acquired are illustrated in Fig. 10.

Discussion

The 19 Bayesian Inference (BI) trees of T9SS protein components exhibit monophyletic clades for all major classes under Bacteroidetes with a strong support for the monophyletic clades or its subclades (Figs. 2-6). Similar to the 19 BI trees of T9SS protein components, the BI tree of 16S rRNA also exhibits monophyletic clades for all major classes under Bacteroidetes with a strong support (Fig. 7). 16S rRNA has been extensively used in phylogenetic analysis for the purpose of evolutionary comparison and classification. The reliability of this approach lies on the assumption that 16S rRNA gene undergoes hierarchical and unidirectional evolution and no gene transfer of 16S rRNA occurs between species (Karlsson et al. 2011). Due to the advantages that 16S rRNA gene has such as ubiquitous gene in bacterial genomes, easily sequenced, and widely available in public sequence databases, the current universal tree of life is based on the phylogeny of this gene (Winker & Woese, 1991; Coutinho et al., 1999; Pylro et al., 2012). That assumption has been challenged due to the presence of multiple copies of 16S rRNA in a bacterial genome and the 16S rRNA genes from operons in the same genome are rather distinct which might suggest such gene might have undergo horizontal gene transfer (Pei et al., 2010; Karlsson et al., 2011). However, the extent of 16S rRNA evolution remains considerably less compared to the other genes in the bacterial genome (Espejo & Plaza, 2018). Thus, 16S rRNA remains relevant for the purpose of evolutionary comparison and classification.

It is expected that the BI trees of T9SS protein components would exhibit similar phylogeny with the BI tree of 16S rRNA. However, the BI trees of T9SS protein components exhibit inconsistent positions of terminal nodes from minor classes among themselves and the phylogeny for the minor classes that deviates from the phylogeny exhibited by BI tree of 16S rRNA (Figs. 2-7). Hence, the minor classes under Bacteroidetes are excluded in the comparison between the 20 BI trees of T9SS protein components. This might arise due to insufficient taxa from minor classes provided to construct those BI trees. Hence, less information is provided which is insufficient to fully resolve the phylogeny of minor classes. As more T9SS-acquiring species from minor classes are sequenced later on, probably the phylogeny of T9SS protein components will be more resolved (Alvizu et al., 2018).

Different from the other 19 BI trees of T9SS protein components, the BI tree of PorR does not exhibit monophyletic clades for all major classes under Bacteroidetes (Figs. 2-6). The presence of a strong support (posterior probability value > 0.95) as denoted by the black branch leading to the top half of BI tree of PorR (Fig. 3C) suggests that there is a strong support for the phylogeny exhibited by BI tree of PorR deviates from the phylogeny based on 16S rRNA sequence (Fig. 7). Thus, there is a possibility that *porR* gene is subjected to horizontal transfer hence causing deviation from the expected phylogeny (Pylro et al., 2012). Hirt, Schlievert & Dunny have demonstrated that virulence factor and antibiotic resistance genes could be horizontally transferred (Hirt, Schlievert & Dunny, 2002). Hence, this suggests the possibility that *porR* gene that encodes one of the virulence factors produced by *P. gingivalis* can be horizontally transferred (Shoji et al., 2002; Shoji et al., 2014).

The arrangement of *porR* and its neighbouring genes in the *P. gingivalis* ATCC 33277 genome is identified in order to support the possibility that *porR* is horizontally transferred. *P. gingivalis* ATCC 33277 genome is chosen because many gene orthologs that involved in A-LPS biosynthesis have been identified in this genome (Shoji et al., 2018). *porR* and its neighbouring genes are found to be flanked by insertion sequences (IS5 family transposons) (Fig. 8). The IS5 family transposons (cyan rectangles) contain a single open reading frame that encodes for IS5 family transposase that cleaves the 12 bp inverted repeats (purple triangles) that flanked the insertion sequences (Fig. 8). The 12 bp inverted repeats show imperfect homology to each other with the consensus sequence: GAGACCTTTG[CA]A. Both of the IS5 family transposons are ~1300 bp in length. These features are typical of IS5 family transposons (Mahillon & Chandler, 1998; Naito et al., 2008). The intervening DNA segment and both IS5 family transposons that flanked it might form a composite transposon where the cleaving action of IS5 family transposases on inverted repeats can mobilise the intervening DNA segment that contains *porR* gene and possibly subjected it to conjugative transfer (Thomas & Nielsen, 2005; Brochet et al., 2009). The length of the composite transposon is ~70 kbp. However, it is also possible for IS5 family transposase to cleave the inverted repeat directly downstream of *rfa* (PGN_1255) (Fig. 8) which will reduce the length of composite transposon to ~47 kbp. It has been reported that a transposon of ~47 kbp in length is able to undergo both transposition and conjugation processes (Brochet et al., 2009). Hence, it might be possible for composite transposon of such length to undergo transposition and subsequently being horizontally transferred via bacterial conjugation.

The intervening DNA segment contains seven genes that involved in the biosynthesis of A-LPS (Fig. 8). Both *porR* (PGN_1236) and *ugdA* (PGN_1243) genes are involved in Wbp pathway that is important for biosynthesis of di-acetylated glucuronic acid that is the structural sugar of A-LPS (Shoji et al., 2002; Shoji et al., 2014). The *porS* gene (PGN_1235), which is an O-antigen flippase, and *wzy* gene (PGN_1242), which is an O-antigen polymerase, are involved in the assembly of A-LPS at the periplasmic side of bacterial IM (Shoji et al., 2013). *gtfB* (PGN_1251) and *gtfE* (PGN_1240) glycosyltransferases genes are involved in the biosynthesis of sugar moiety of A-LPS. *rfa* (PGN_1255) glycosyltransferase gene is involved in the biosynthesis of lipid A-core moiety of A-LPS (Shoji et al., 2018). However, there are other genes that involved in the biosynthesis of A-LPS and they are spread out throughout the genome (Shoji et al., 2018). Usually, genes that are co-regulated and involved in a similar pathway are clustered in a single operon (Yanofsky & Lennox, 1959; Osbourn & Field, 2009). Thus, it is possible that the other genes do not form a cluster with the seven genes that are identified to be flanked by insertion sequences (IS5 family transposons) because they are not co-regulated.

It is possible that those seven genes might be co-transferred via horizontal gene transfer. Thus, phylogenetic analysis is performed for the protein alignment of UgdA that is encoded by *ugdA* that, together with *porR*, are involved in the Wbp pathway and are co-localised in the intervening DNA segment flanked by IS5 family transposons (Fig. 8). The BI tree of UgdA (Fig. 9B) is constructed to be compared with the BI tree of PorR (Fig. 9A). Different to the 19 BI trees of T9SS protein components, both BI trees do not exhibit monophyletic clades for all major

classes under Bacteroidetes. They also exhibit similar topology where four similar clusters (I, II, III, and IV) with a strong support (denoted by a black branch leading to the cluster) have been identified in both BI trees. Cluster I consists of terminal nodes from Flavobacteriia and a few terminal nodes from other classes. Cluster II consists of terminal nodes from Porphyromonas, Tannerella, and Parabacteroides genera. Cluster III consists of terminal nodes from Rufibacter and Hymenobacter genera. Cluster IV consists of terminal nodes from Prevotella, Bacteroides, Proteiniphilum and other genera. These four clusters exhibit similar relative positions to each other in both BI trees (e.g. cluster I is closer to cluster II than the other clusters and cluster III is closer to cluster II than the other clusters). However, due to the differences in branch lengths between both BI trees, they look slightly different as the upper part of UgdA tree (Fig. 9B) appears more elongated than the upper part of PorR tree (Fig. 9A) while the lower part of UgdA tree (cluster I) appears more shorten than the lower part of PorR tree (cluster I).

Other than the BI tree of PorR, the BI trees of other 19 T9SS protein components also exhibit evidence of horizontal gene transfer perhaps between classes under Bacteroidetes. As listed in Table S1, there are terminal nodes that are out of its expected monophyletic clades in the BI trees of those components that suggest the genes that encode them might be horizontally transferred. In theory, the common ancestral species of a monophyletic clade for a class under Bacteroidetes passes the gene that encodes T9SS protein component to its descendant species. Thus, the descendant species that are out of its expected monophyletic clades most likely acquire that gene from the common ancestral species of a monophyletic clade from another class that could be interpreted as a horizontal gene transfer between classes under Bacteroidetes (Thomas & Nielsen, 2005; Brochet et al., 2009). It is interesting to highlight that there are species that frequently have their corresponding terminal nodes in those 19 BI trees out of its expected monphyletic clades (Figs. 2-6) such as *F. taffensis* DSM 16823, bacterium L21-Spi-D4, *O. hongkongensis* DSM 17368, and *D. orientale*. Thus, it likely that those bacteria frequently acquire their T9SS components through horizontal gene transfer. However, for the genes that encode those 19 components, they might undergo horizontal gene transfer less frequently compared to *porR* that causes most of the terminal nodes of BI trees of those components are still clustering according to their respective classes. It might be because the intervening DNA segment that contains *porR* gene is easily exchanged between bacteria under Bacteroidetes due to the present of insertion sequences (IS5 family transposons) that flanking it (Fig. 8).

T9SS is made up of various protein components that formed the regulation, translocation, energetic, and modification components. Currently, the secretion system is primarily found in bacteria under Bacteroidetes phylum (Abby et al., 2016). Bacteria from classes under Bacteroidetes (Bacteroidia, Flavobacteriia, Cytophagia, Chitinophagia, Sphingobacteriia, Saprospiria, Incertae sedis, and unclassified) are found to acquire T9SS protein components (Figs. 2-6). However, not all of them acquire the 20 components that have been reported (Sato et al., 2010; Lasica et al., 2017). As shown in the 20 BI trees of T9SS protein components (Figs. 2-6), only bacteria under Bacteroidia, Flavobacteriia, and Chitinophagia acquired the 20 components investigated. The bacteria under Cytophagia only acquired 19 components (except

PorN). The bacteria under Saprospiria only acquired 18 components (except PorL and PorG). The bacteria under Sphingobacteriia only acquired 17 components (except PorQ, PorU, and PorZ). The bacteria under unclassified only acquired 17 components (except PorN, PorU, and PorG). The bacteria under Incertae sedis only acquired 11 components (PorQ, PorR, Sov, PorU, PorV, PorX, PorY, PorZ, SigP, Omp17, and PorF). It is interesting to note that PorU, PorZ, and PorQ formed the modification components of T9SS. Thus, Sphingobacteriia does not acquire the components that performed post-translational modifications on T9SS cargo proteins such as cleavage of CTD and A-LPS glycosylation. Perhaps, T9SS acquired by Sphingobacteriia does not cleave the CTD of cargo protein and glycosylate it with A-LPS but leave the cargo protein bounded to PorV after it is translocated to bacterial cell surface by Sov. Other possible explanation is that Sphingobacteriia does have proteins that performed the functions of missing protein components. However, those proteins exhibited limited sequence similarity with any currently known T9SS protein component. Thus, they could not be detected by homology searching method used in this work. This explanation could also be applied for other species of bacteria under Bacteroidetes that do not acquire the homologs of 20 T9SS components.

This work has found other species under Chitinophagia, Saprospiria, and unclassified that acquired homologs of T9SS components that, to our knowledge, might not have been reported (McBride & Zhu, 2013). Those other species and the homologs of T9SS components they acquired are shown in Fig. 10. This identification might be due to the analysis that has been performed might cover more bacterial species than the previous work as more bacterial genomes have been completely sequenced in the past few years.

Conclusions

The objective of this work is to investigate the phylogenetic relationship among putative members of 20 T9SS component protein families (Emrizal & Muhammad, 2018). The Bayesian Inference (BI) trees for 19 T9SS protein components exhibit monophyletic clades for all major classes under Bacteroidetes with a strong support for the monophyletic clades or its subclades which is consistent with phylogeny exhibited by the constructed BI tree of 16S rRNA. However, the BI tree of PorR is different from the other 19 BI trees of T9SS protein components as it does not exhibit monophyletic clades for all major classes under Bacteroidetes. There is a strong support for the phylogeny exhibited by BI tree of PorR deviates from the phylogeny based on 16S rRNA sequence. Thus, there is a possibility that *porR* gene is subjected to horizontal transfer as it is known that virulence factor gene could be horizontally transferred. Seven genes that involved in the biosynthesis of A-LPS that includes *porR* are found to be flanked by insertion sequences (IS5 family transposons). This suggests that the intervening DNA segment that contains *porR* gene can be transposed and subjected to conjugative transfer. Thus, the seven genes might be co-transferred via horizontal gene transfer. Similar to the BI tree of PorR, the BI tree of UgdA does not exhibit monophyletic clades for all major classes under Bacteroidetes (both are a part of the seven genes). Both BI trees also exhibit similar topology where the four

identified clusters with a strong support have similar relative positions to each other in both BI trees. Other than the BI tree of PorR, the BI trees of other 19 components also exhibit evidence of horizontal gene transfer. However, for the genes that encode those 19 components, they might undergo horizontal gene transfer less frequently compared to *porR* because the intervening DNA segment that contains *porR* is easily exchanged between bacteria under Bacteroidetes due to the presence of insertion sequences (IS5 family transposons) that flanking it. This work also found other species under Chitinophagia, Saprospiria, and unclassified that acquired T9SS component protein homologs that, to our knowledge, might not have been reported.

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Figure 1

T9SS protein components on the inner membrane (IM) and outer membrane (OM) of *Porphyromonas gingivalis*.

The protein components with known functions are represented by coloured structures. The pathway for cargo protein gingipain (RgpB) translocation and modifications by T9SS is illustrated. The regulation of the pathway by the protein components is also exhibited.

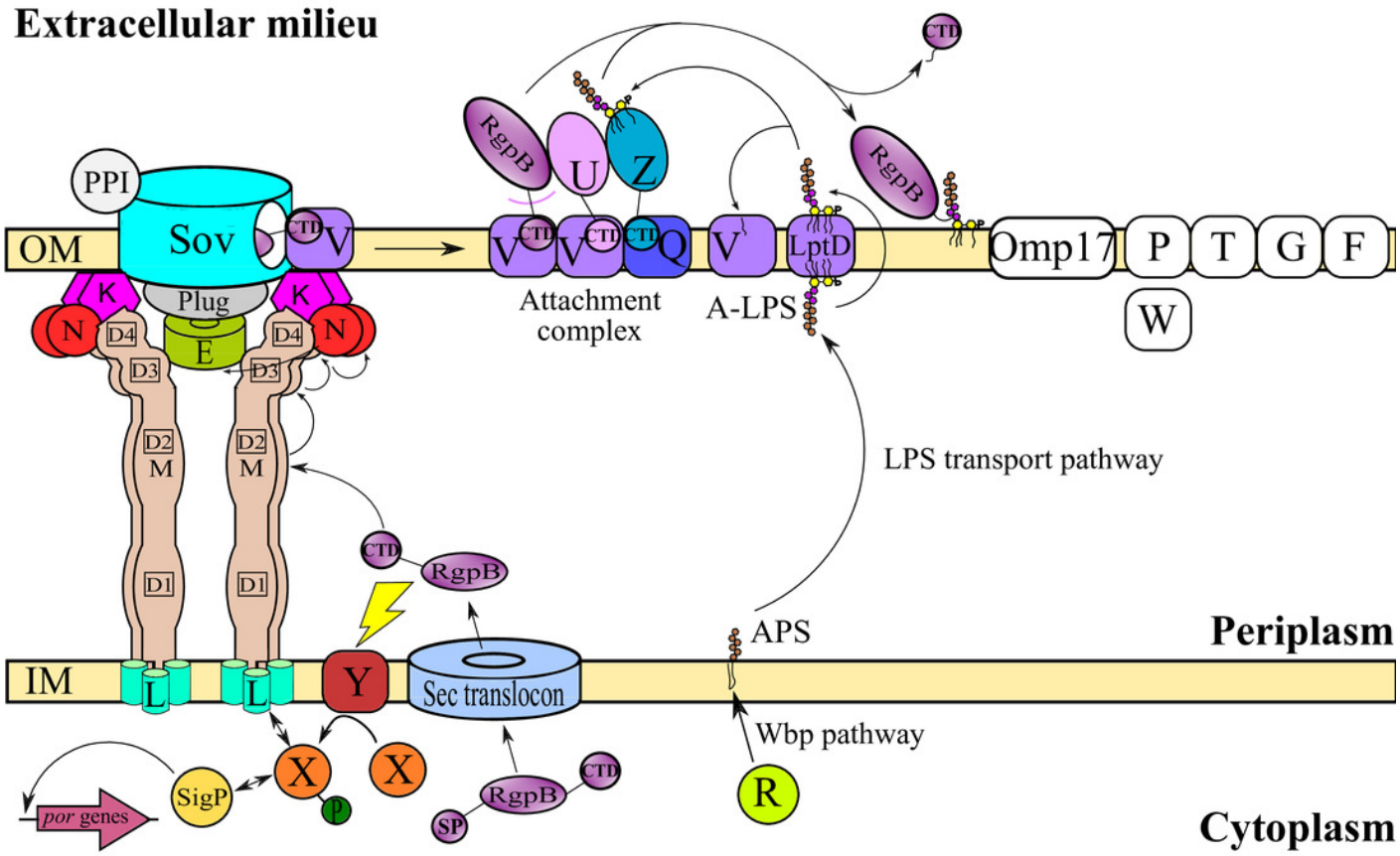

Figure 2

The Bayesian Inference (BI) phylogenetic trees of T9SS protein components (PorK, PorL, PorM, and PorN).

(A) BI tree of PorK. (B) BI tree of PorL. (C) BI tree of PorM. (D) BI tree of PorN. The terminal nodes are labelled with coloured circles that represent the classes under Bacteroidetes that each protein homolog belongs to. The classes represented by each colour are provided in the legend inside the figure. The branches with strong support (posterior probability value > 0.95) are coloured in black. Otherwise, the branches are coloured in red. The solid curve denotes a monophyletic clade that formed by terminal nodes that belong to the same class under Bacteroidetes. The dashed curve denotes a strong support for the monophyletic clade or its subclade. The colour of curve represents the class of terminal nodes that form the clade. The classes represented by each colour are shown in the legend inside the figure.

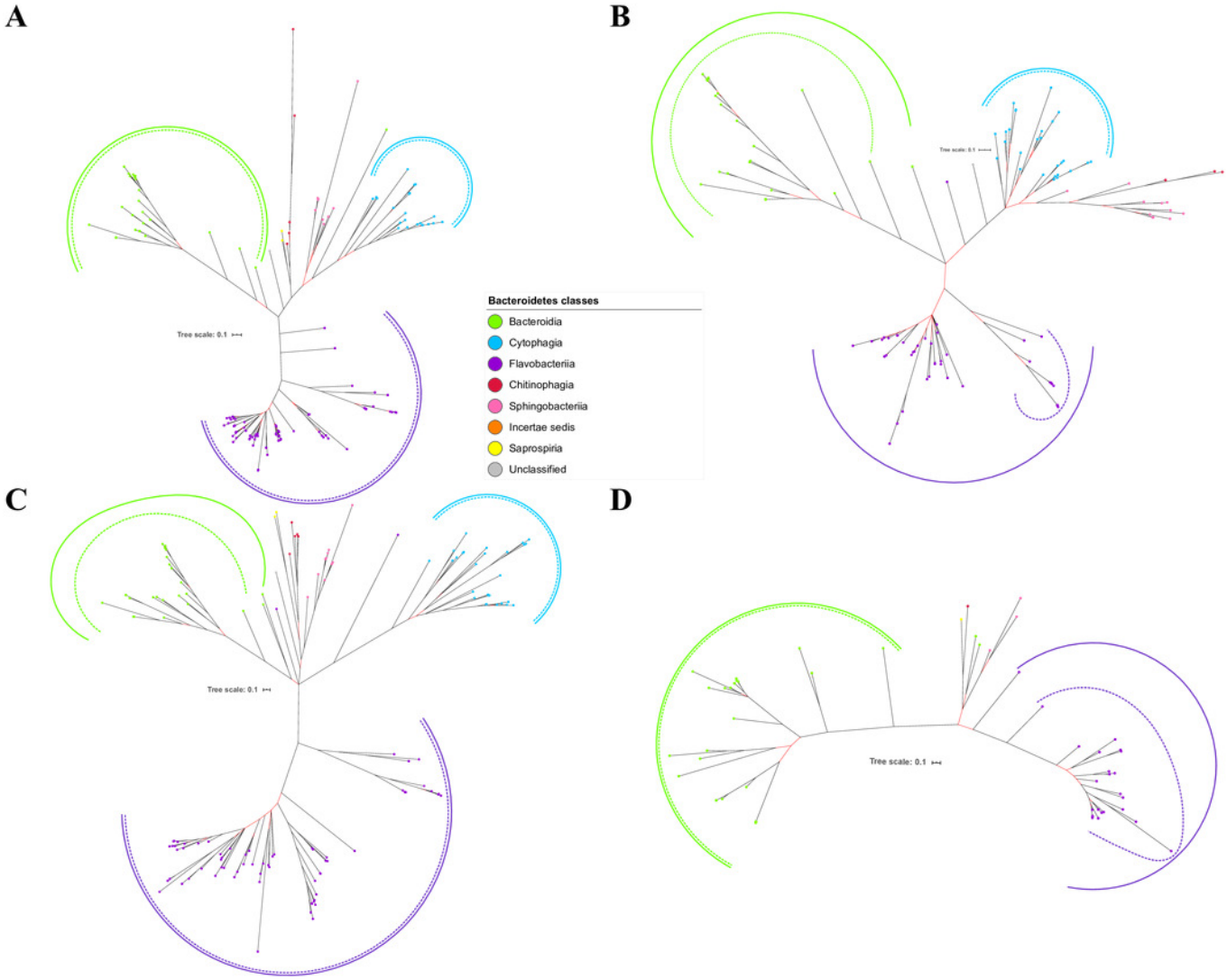
A
B
C
D
Bacteroidetes classes
Bacteroidia
Cytophagia
Flavobacteriia
Chitinophagia
Sphingobacteriia
Incertae sedis
Saprospiria
Unclassified
Tree scale: 0.1

Figure 3

The Bayesian Inference (BI) phylogenetic trees of T9SS protein components (PorP, PorQ, PorR, and Sov).

(A) BI tree of PorP. (B) BI tree of PorQ. (C) BI tree of PorR. (D) BI tree of Sov.

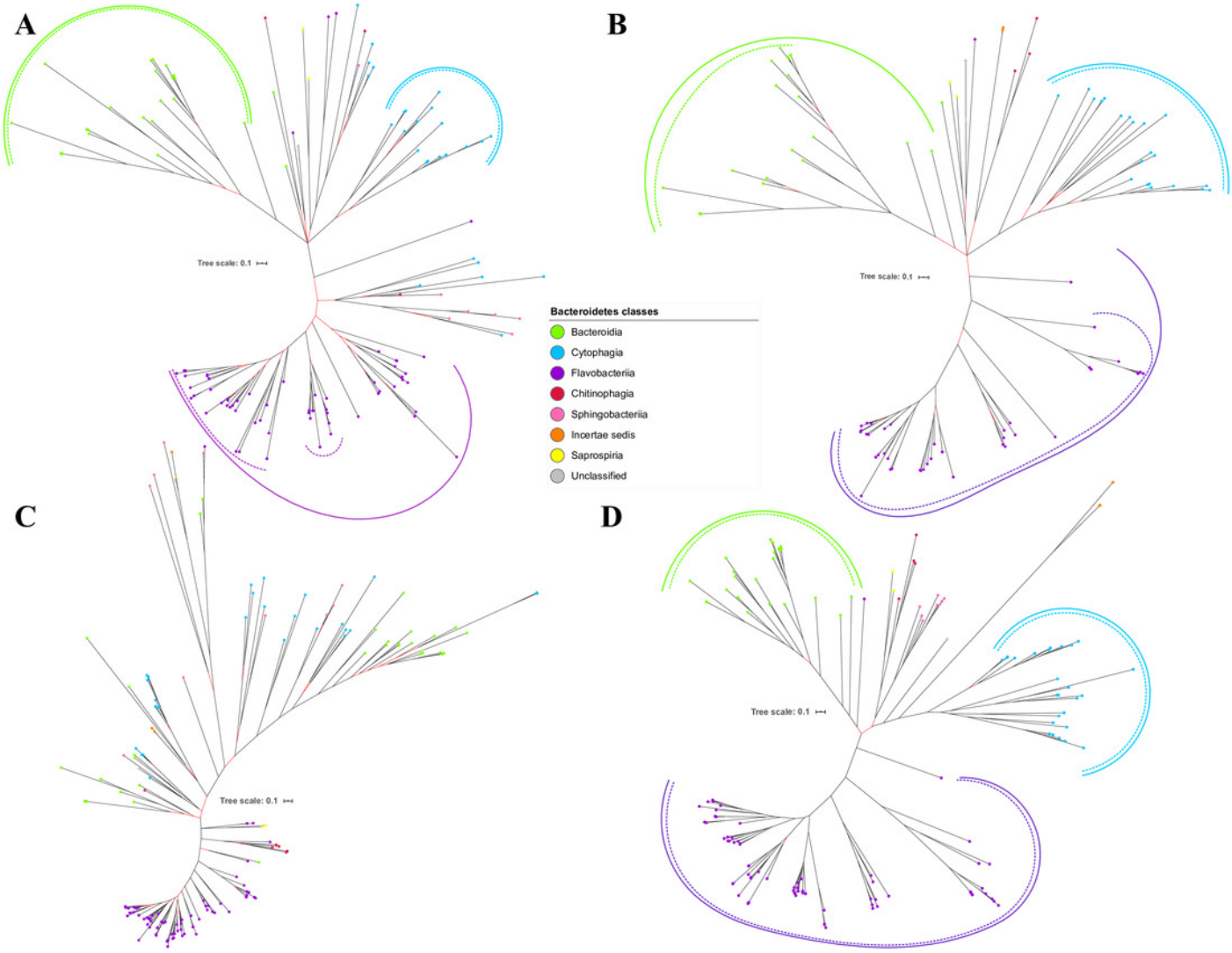

Figure 4

The Bayesian Inference (BI) phylogenetic trees of T9SS protein components (PorT, PorU, PorV, and PorW).

(A) BI tree of PorT. (B) BI tree of PorU. (C) BI tree of PorV. (D) BI tree of PorW.

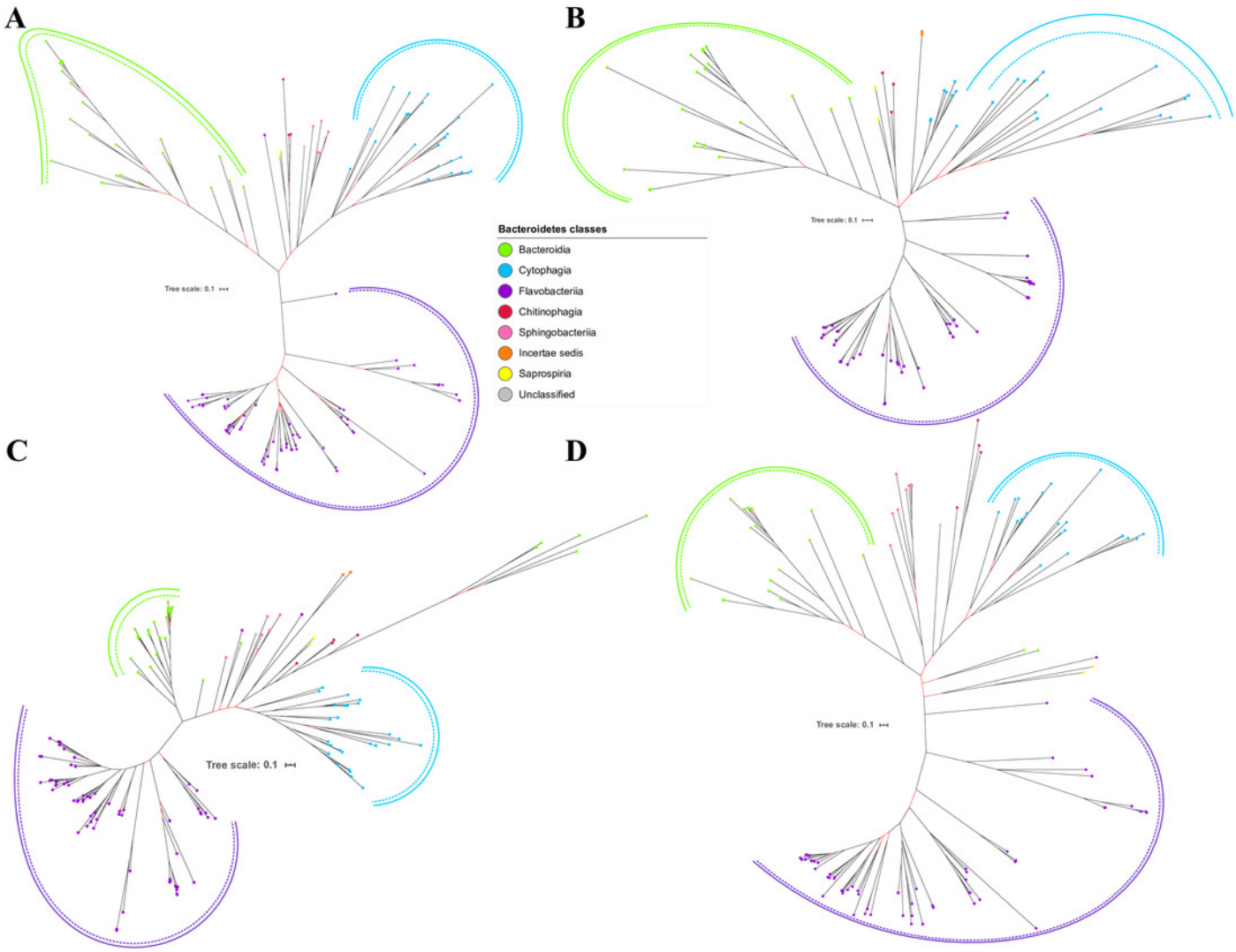

Figure 5

The Bayesian Inference (BI) phylogenetic trees of T9SS protein components (PorX, PorY, PorZ, and SigP).

(A) BI tree of PorX. (B) BI tree of PorY. (C) BI tree of PorZ. (D) BI tree of SigP.

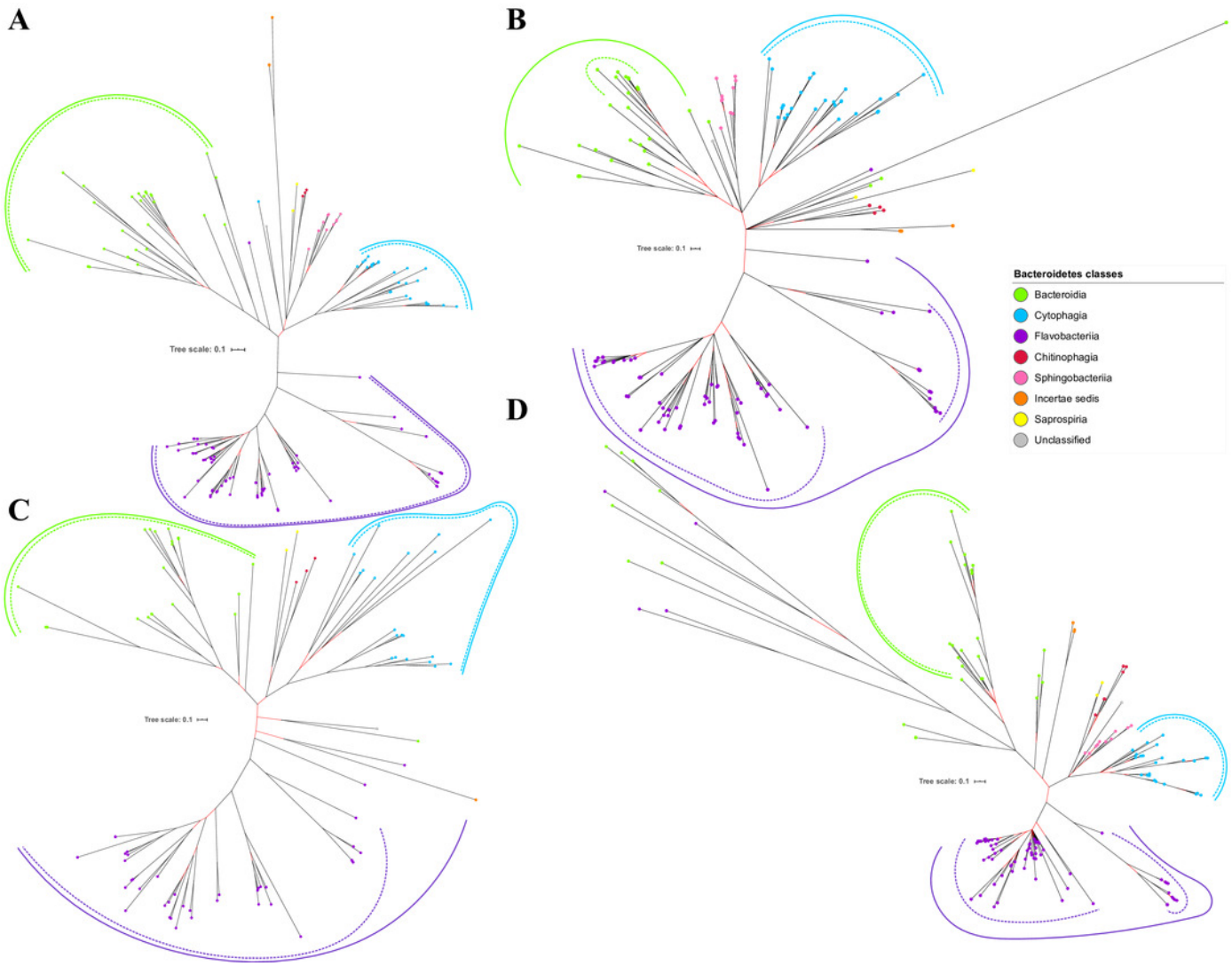

Figure 6

The Bayesian Inference (BI) phylogenetic trees of T9SS protein components (Omp17, PorE, PorF, and PorG).

(A) BI tree of Omp17. (B) BI tree of PorE. (C) BI tree of PorF. (D) BI tree of PorG.

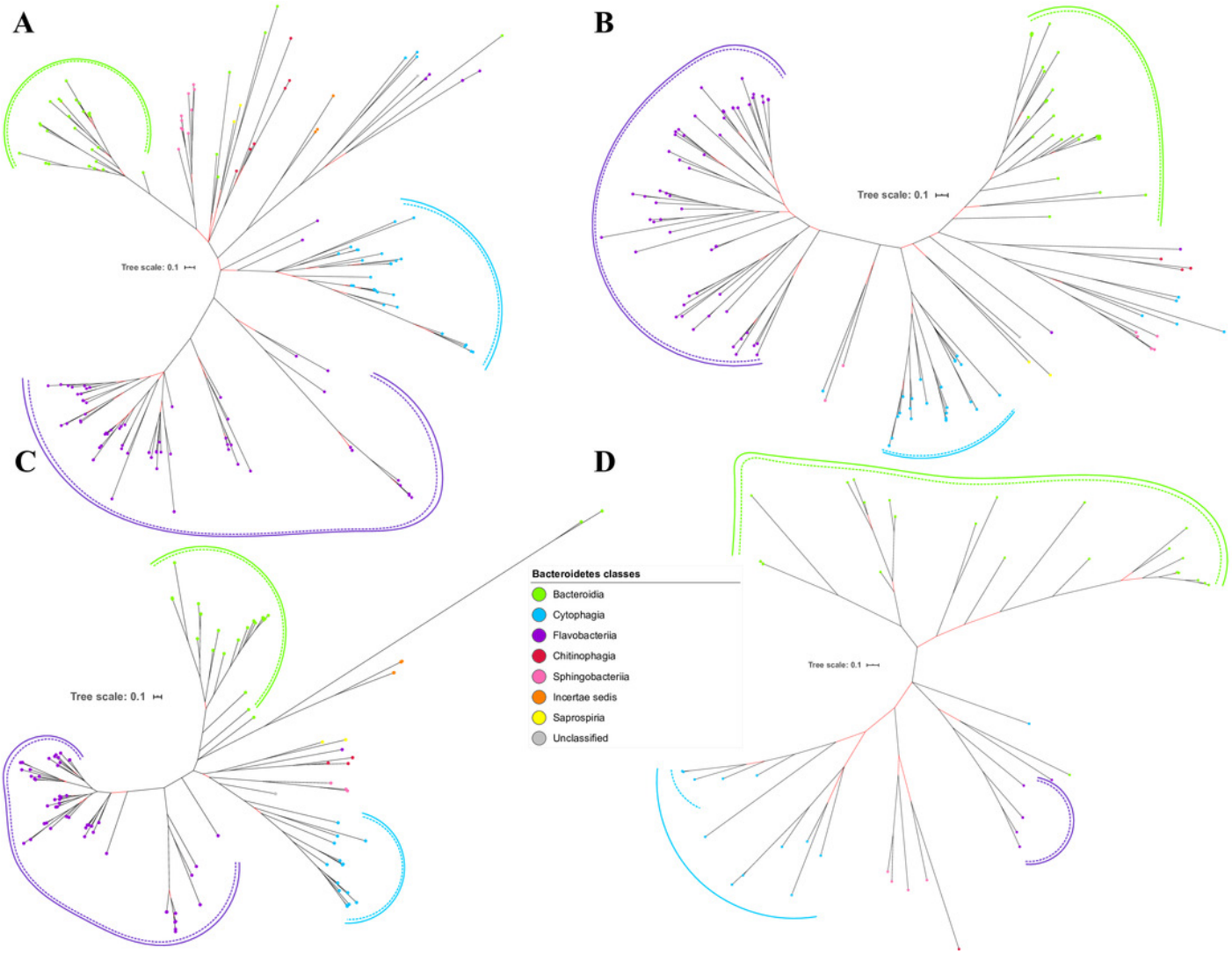

Figure 7

The Bayesian Inference (BI) phylogenetic tree of 16S ribosomal RNA (rRNA).

The BI tree of 16S rRNA exhibits monophyletic clades where each clade consists of terminal nodes of the same colour that denotes that they belong to the same class under Bacteroidetes. There is a high support (posterior probability value > 0.95) for each monophyletic clade indicates by the black branch leading to each clade. The solid and dashed green, purple, and blue curves indicate there is a strong support for the monophyletic clades of Bacteroidia, Flavobacteriia, and Cytophagia classes respectively.

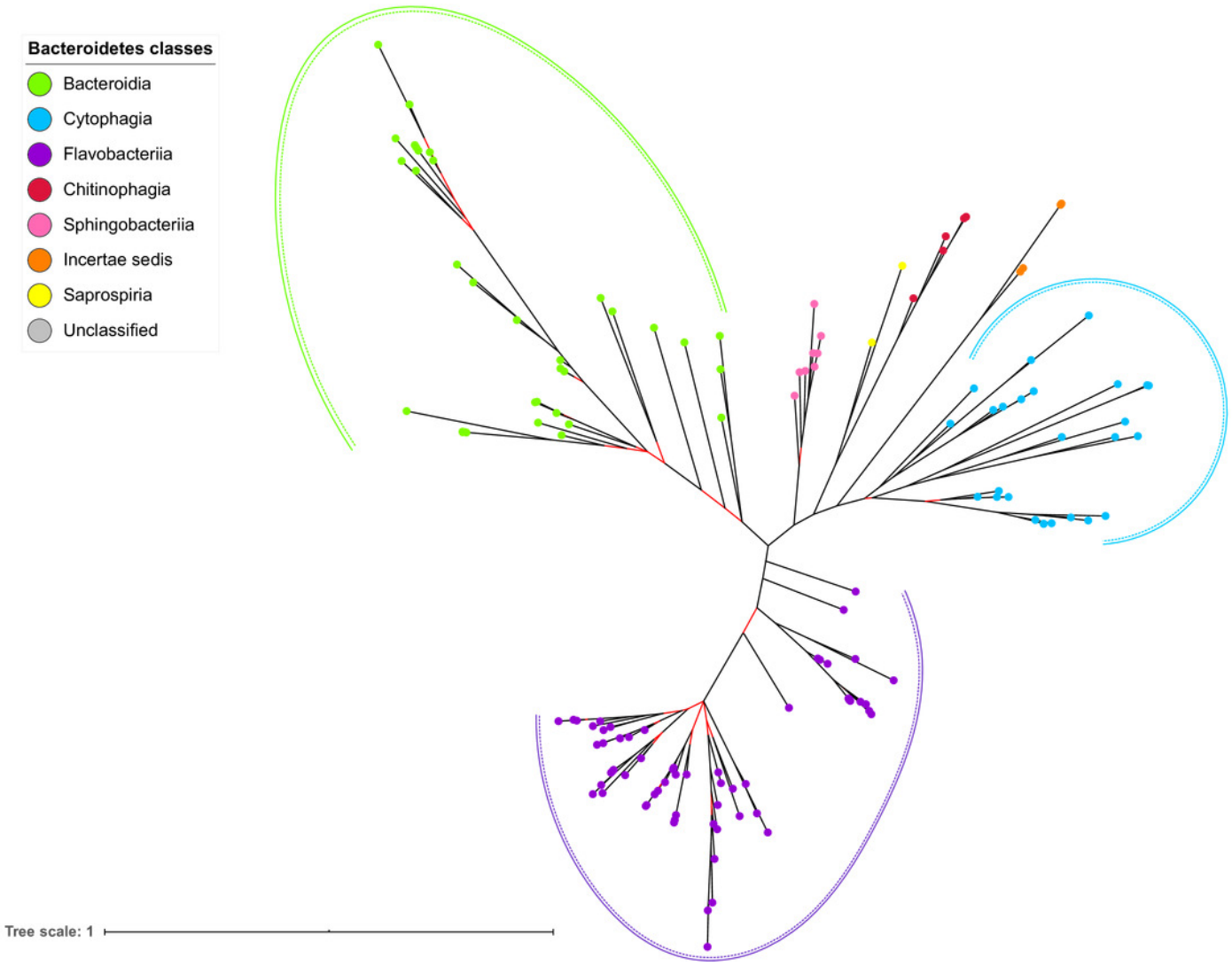

Figure 8

The arrangement of *porR* and its neighbouring genes in *P. gingivalis* ATCC 33277 genome.

porR (PGN_1236) and its neighbouring genes are flanked by IS5 family transposons that formed a composite transposon of 70 kbp in length. The genes that involve in biosynthesis of A-LPS are represented by yellow rectangles while the gene that does not involve is represented by brown rectangle. The genes for hypothetical proteins are represented by white rectangles. The genes for IS5 family transposases are represented by cyan rectangles. The purple triangles represented 12 bp inverted repeats that flanked the genes for IS5 family transposases. Name of proteins encoded by the genes are shown under rectangles that represented the genes. The slashes indicated gaps in the genome.

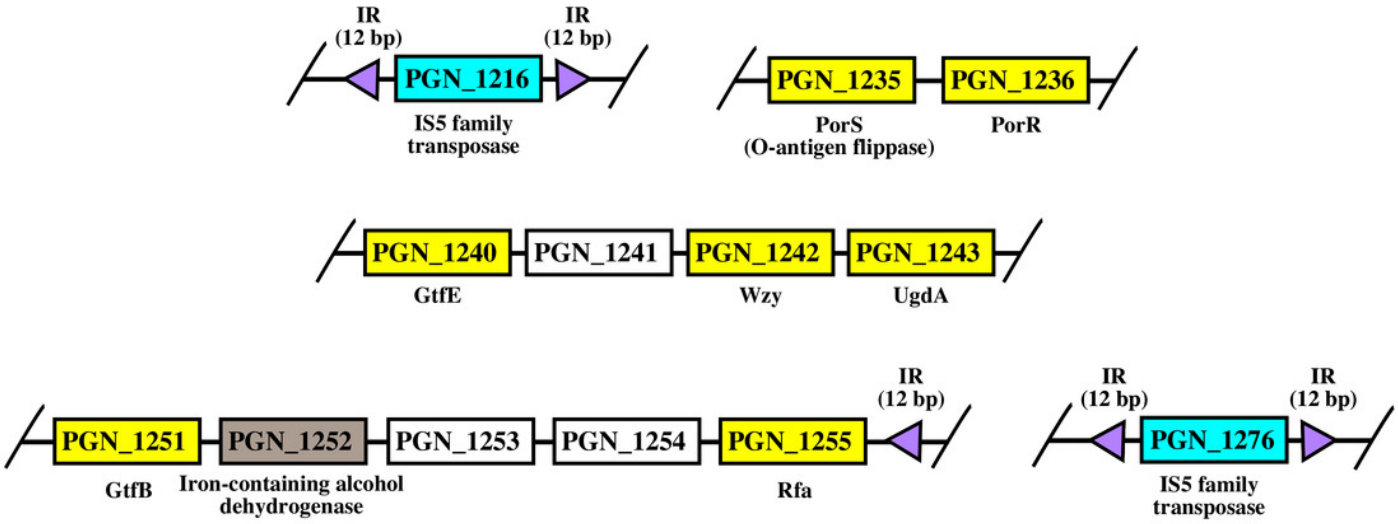
IR
(12 bp)
IR
(12 bp)
PGN_1216
IS5 family
transposase
PGN_1235
PGN_1236
PorS
(O-antigen flippase)
PorR
PGN_1240
PGN_1241
PGN_1242
PGN_1243
GtfE
Wzy
UgdA
IR
(12 bp)
PGN_1251
PGN_1252
PGN_1253
PGN_1254
PGN_1255
GtfB
Iron-containing alcohol
dehydrogenase
Rfa
IR
(12 bp)
IR
(12 bp)
PGN_1276
IS5 family
transposase

Figure 9

Comparison between the Bayesian Inference (BI) tree of UgdA with BI tree of PorR.

The BI tree of UgdA (A) does not exhibit monophyletic clades for all major classes under Bacteroidetes which is similar to the BI tree of PorR (B). Both BI trees of UgdA and PorR also exhibit similar topology. Both trees exhibit cluster I (solid purple curve) that primarily consists of terminal nodes of Flavobacteriia and a few terminal nodes from other classes. Both trees have cluster II (solid green curve) that consists of terminal nodes of Porphyromonas, Tannerella, and Parabacteroides genera. Both trees acquire cluster III (solid blue curve) that consists of terminal nodes of Rufibacter and Hymenobacter genera. Both trees exhibit cluster IV (solid green curve) that consists of terminal nodes of Prevotella, Bacteroides, Proteiniphilum, and other genera.

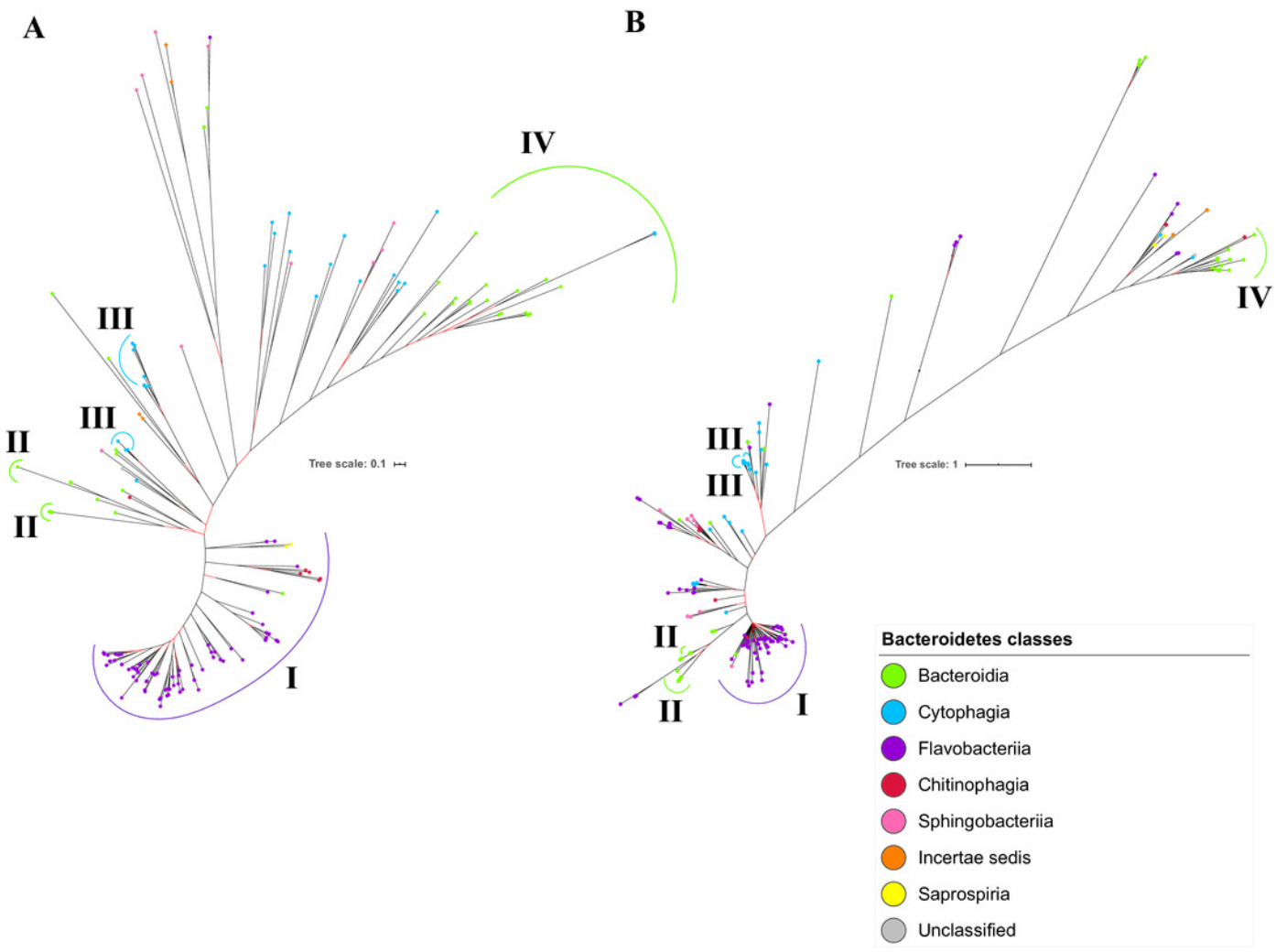
A
IV
III
III
II
II
Tree scale: 0.1
I
B
III
III
II
II
I
IV
Tree scale: 1
Bacteroidetes classes
Bacteroidia
Cytophagia
Flavobacteriia
Chitinophagia
Sphingobacteriia
Incertae sedis
Saprospiria
Unclassified

Figure 10

The species from Chitinophagia, Saprospiria, and unclassified under Bacteroidetes phylum that acquired homologs of T9SS protein components.

The colours of rectangles denote the classes those species belong to. Coloured squares indicate T9SS component homologs acquired by the species where the different colours denote different functions those components performed. White squares indicate T9SS component homologs absent in those species.

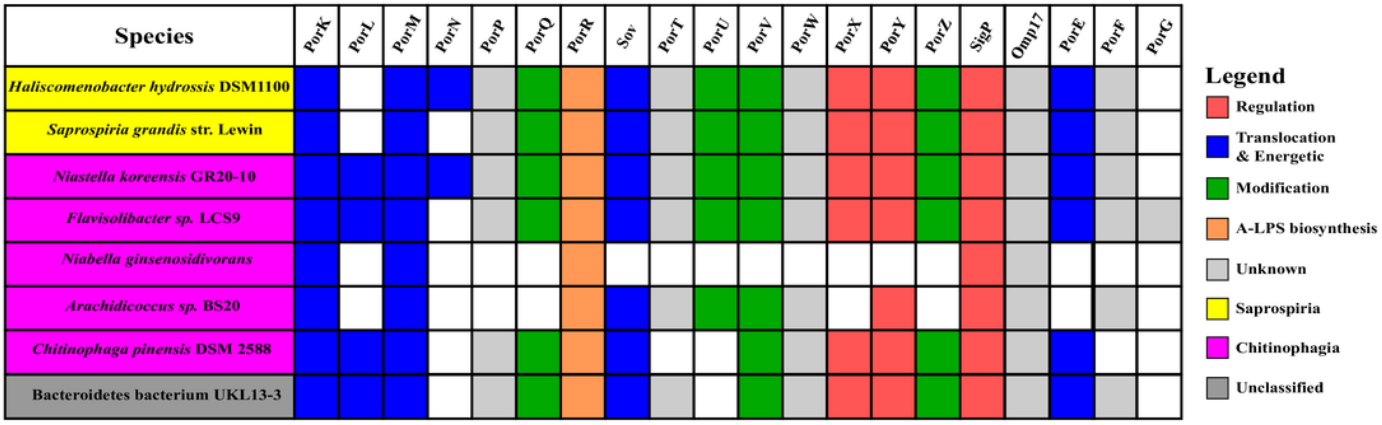

Table 1(on next page)

The characteristics of T9SS component protein alignments and the best amino acid substitution models that have been selected for them.

The characteristics of T9SS component protein alignments such as no. of taxa used to construct the alignments and no. of characters of the alignments are provided. The best amino acid substitution model that has been selected for each alignment is also provided. The definition of parameters of the best amino acid substitution models are provided in the footnote.

Alignment	No. of taxa	No. of characters	Model
Omp17	180	245	LG + G + F
PorE	137	793	LG + G + I
PorF	121	829	LG + G + I + F
PorG	55	487	LG + G + I
PorK	153	561	LG + G + I
PorL	123	281	LG + G + I
PorM	159	406	LG + G + I + F
PorN	62	267	LG + G + I
PorP	138	281	LG + G + I + F
PorQ	108	358	LG + G + F
PorR	176	471	LG + G + I
PorT	151	202	LG + G + F
PorU	109	919	LG + G + I
PorV	162	360	LG + G + I + F
PorW	137	995	LG + G + I + F
PorX	162	624	LG + G + I
PorY	162	897	LG + G + I
PorZ	102	569	LG + G + I
SigP	177	197	LG + G + I
Sov	159	2704	LG + G + I + F
UgdA	176	460	LG + G + I
16S rRNA	144	1452	GTR + G + I

Note:

- LG + G: LG substitution model matrix with gamma-shaped rate variation across sites and Dirichlet stationary amino acid frequencies
- LG + G + I: LG substitution model matrix with gamma-shaped rate variation across sites with a proportion of invariable sites and Dirichlet stationary amino acid frequencies
- LG + G + I + F: LG substitution model matrix with gamma-shaped rate variation across sites with a proportion of invariable sites and fixed (empirical) stationary amino acid frequencies
- LG + G + F: LG substitution model matrix with gamma-shaped rate variation across sites and fixed (empirical) stationary amino acid frequencies
- GTR + G + I: General Time Reversible (GTR) substitution model matrix with gamma-shaped rate variation across sites with a proportion of invariable sites and Dirichlet stationary nucleotide frequencies