T9GPred: A Comprehensive Computational Tool for the Prediction of Type 9 Secretion System, Gliding Motility and the Associated Secreted Proteins

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ABSTRACT

Type 9 secretion system (T9SS) is one of the least characterized secretion systems exclusively found in *Bacteroidetes* phylum which comprise various environmental and economically relevant bacteria. T9SS plays a central role in bacterial movement termed gliding motility, survival and pathogenicity. However, there are no comprehensive attempts to predict T9SS, gliding motility and proteins secreted via T9SS. In this study, we curated the published evidence and generated protein profiles for the compiled 28 protein components associated with T9SS or gliding motility in *Bacteroidetes*. We leveraged the generated protein profiles to designate 6 proteins (GldK, GldL, GldM, GldN, SprA and SprE) as mandatory components for T9SS and an additional 5 proteins (GldB, GldD, GldH, GldJ and SprT) as mandatory components for gliding motility. Thereafter, we compiled 102 experimentally characterized proteins secreted via T9SS and classified their C-terminal domain (CTD) into 3 types (type A, type B or type C). We generated protein profiles for these CTDs and found that they are unique to proteins secreted via T9SS. We developed a computational tool, **T**ype **9** secretion system and **G**liding motility **Pred**iction (T9GPred) by compiling our findings. From 693 completely sequenced *Bacteroidetes* strains, T9GPred predicted 402 to have T9SS, of which 327 are also predicted to exhibit gliding motility. Additionally, T9GPred predicted the putative secreted proteins from the 402 *Bacteroidetes* strains. The computational tool is available in our GitHub repository: https://github.com/asamallab/T9GPred. The tool and its predicted results are compiled in a web server available at: https://cb.imsc.res.in/t9gpred/.

IMPORTANCE

We report the first comprehensive computational tool which predicts the presence of T9SS or gliding motility, and proteins secreted via T9SS in the members of the *Bacteroidetes* phylum. Using this tool, we showed that T9SS is exclusively found in the *Bacteroidetes*

phylum. Our analysis of the motility components showed that the gliding motility associated with T9SS is uniquely exhibited by members of the *Bacteroidetes* phylum. We also observed that the secretion signal is unique to proteins secreted via T9SS in comparison with other *Bacteroidetes*-specific secretion systems. In a nutshell, the developed computational tool is a novel strategy which provides a systems-level prediction of T9SS for streamlining future experimentation.

KEYWORDS Computational Systems Biology, Bioinformatics, T9SS, Gliding motility, *Bacteroidetes*, Computational prediction tool, Secretion signal

INTRODUCTION

Type 9 secretion system (T9SS) is one of the recently characterized secretion systems found particularly in members of the *Bacteroidetes* phylum (1–4). *Bacteroidetes* is a diverse phylum of bacteria found in different environmental niches including animal gut microbiota, where they are either beneficial or pathogenic based on their secretory factors (5, 6). In some of these *Bacteroidetes* strains, T9SS has been experimentally identified to play a central role in movement of the bacteria (1, 7–9), assimilation of ions (10), and in the secretion of various polysaccharide degrading enzymes (10, 11), adhesins (12) and virulent proteins (2, 13). Additionally, T9SS is found to be essential for the growth and survival of some species (13–15). Thus, identification of T9SS is imperative to understand the underlying microbial activity.

T9SS was initially characterized in two different *Bacteroidetes* species, *Porphyromonas gingivalis* (human periodontal pathogen) (1, 16) and *Flavobacterium johnsoniae* (1). In particular, McBride and colleagues had extensively characterized the protein components associated with gliding motility of *F. johnsoniae* (17, 18) and later found a large overlap with the T9SS components (3). Additional experiments proved that the

gliding motility is facilitated by secretion of adhesins through T9SS (19, 20). Further, several groups have identified functionalities of the core protein components of T9SS namely, GldK, GldL, GldM, GldN and SprA (21–24). James *et al*. (25) have shown that the GldLM complex is conserved across members of the *Bacteroidetes* phylum and is mediated through proton flow to drive the secretion via T9SS (22, 26). There are other such independent studies focussed on T9SS or gliding motility in members of the *Bacteroidetes* phylum (7, 8, 27), but there is scarce attempt toward systems-level understanding of the components associated with T9SS or gliding motility.

Secretion via T9SS is a two-step process: (i) transport of protein from cytoplasm to periplasm via Sec export pathway mediated by N-terminal domain; (ii) transport of protein from periplasm to extracellular space via T9SS mediated by C-terminal domain (CTD) (13, 28). Kulkarni *et al.* (29) have experimentally proved that the N-terminal domain and the CTD signal are necessary and sufficient for the secretion of protein via T9SS. Additionally, they have identified different types of CTD. While efforts have been made to characterize the secreted proteins, there have been limited attempts to identify the CTD types and predict such secreted proteins.

Earlier, Abby *et al.* (4, 30) had developed a computational tool, TXSScan to identify different secretion systems including T9SS across bacteria. However, TXSScan is neither specific to T9SS nor does it predict gliding motility or proteins secreted via T9SS. Therefore, in this study, we built a prediction tool which is dedicated to T9SS, gliding motility and the proteins secreted via T9SS. Initially we compiled the proteins associated with T9SS or gliding motility from published literature and generated Hidden Markov Model (HMM) profiles for each protein. We leveraged the experimental knowledge to identify the key protein components and built a computational tool for prediction of T9SS or gliding motility across members of the *Bacteroidetes* phylum. Note that, we have included gliding motility

prediction along with that of T9SS as some *Bacteroidetes* strains having T9SS do not exhibit gliding motility (1, 13). Previously, Veith *et al.* (31) have used HMM based approaches to predict proteins secreted via T9SS. However, their prediction model did not account for different types of CTD. Here we leveraged the different CTD types and their motifs from published literature to generate HMM profiles and built a computational pipeline for the prediction of proteins secreted via T9SS. Finally, we developed a computational tool, **T**ype **9** secretion system and **G**liding motility **Pred**iction (T9GPred), which is accessible at https://github.com/asamallab/T9GPred. We also compiled the tool and its predictions into a web server namely T9GPred, which is accessible at https://cb.imsc.res.in/t9gpred/.

RESULTS

Identification of 28 protein components associated with T9SS or gliding motility among members of the *Bacteroidetes* phylum

We compiled 23 protein components associated with T9SS (**Table S1**), 22 protein components associated with gliding motility (**Table S2**), and 102 proteins secreted via T9SS (secreted proteins) (**Table S3**) in *Bacteroidetes* strains from published literature (Methods; **Figure 1**). We observed that there is a large overlap of 17 proteins between the proteins associated with T9SS and gliding motility, which is in line with the previous studies reporting conclusive evidence of a link between T9SS and gliding motility in *Bacteroidetes* strains (1, 3). In total, we identified 28 proteins associated with either T9SS or gliding motility among members of the *Bacteroidetes* phylum, which is a much larger set in comparison with the set of 11 proteins used by TXSScan (4, 30) for their prediction.

Key protein components of T9SS and gliding motility

During the compilation of associated protein components (**Figure 1**), we observed that not all the components are present in every bacterium of the *Bacteroidetes* phylum that

have experimental evidence for T9SS or gliding motility (**Figure 2**). Therefore, we were interested in identifying the key components which are mandatory for a functional T9SS or exhibiting gliding motility. We generated HMM profiles for the 28 identified protein components (Methods; **Supplemental Information**) and checked their presence in 30 *Bacteroidetes* strains that have experimental evidence for the presence of T9SS or gliding motility (**Table S4**). We observed that proteins GldK, GldL, GldM, GldN, SprA, SprE and SprT are always present in the *Bacteroidetes* having T9SS in comparison with those which lack T9SS (**Figure 2**). The proteins GldL and GldM form an inner membrane proton-driven motor that drives protein secretion via T9SS (**Figure 3**) (22, 26). The GldK-GldN ring and SprE facilitate energy transduction from the inner membrane GldLM motor to the outer membrane translocon SprA for the secretion of proteins to the extracellular space (**Figure 3**) (21, 22, 24, 32). SprT aids in the maturation of the secreted protein but its role in the secretion mechanism is not yet known (16). Therefore, we designated GldK, GldL, GldM, GldN, SprA and SprE (6 proteins) as the mandatory protein components to predict the presence of T9SS.

In addition to the 6 T9SS mandatory components, we observed that proteins GldB, GldD, GldH, GldJ and SprT are present in the *Bacteroidetes* strains exhibiting gliding motility in comparison with those which lack gliding motility (**Figure 2**). Importantly, McBride *et al.* (3) had earlier characterized the presence of these proteins in *Bacteroidetes* strains exhibiting gliding motility. Therefore, we designated the 6 T9SS mandatory components along with GldB, GldD, GldH, GldJ and SprT (a total of 11 proteins) as mandatory protein components to predict the presence of gliding motility. We designated the remaining 17 proteins as accessory components.

Computational tool for prediction of T9SS or gliding motility across *Bacteroidetes* strains

We leveraged the generated HMM profiles to check for presence of the 28 proteins associated with T9SS or gliding motility in the proteome of 693 completely sequenced *Bacteroidetes* strains (Methods; **Table S5**). If a bacterium contains all 6 T9SS mandatory components in its proteome, we classify it as having T9SS. In addition, if a bacterium contains all the 11 gliding motility mandatory components, we classify it as having gliding motility. Using this criteria, we developed a tool using a python program compiling the generated HMM profiles for 28 proteins to predict the presence of T9SS or gliding motility across 693 *Bacteroidetes* strains. Our tool identified 402 *Bacteroidetes* strains to have T9SS, of which 327 were found to also have gliding motility. Typically, we observed that 19 of the 28 proteins are present in the 402 *Bacteroidetes* strains predicted to have T9SS (**Figure S1a**), while 22 of the 28 proteins are present in the 327 *Bacteroidetes* strains predicted to have gliding motility (**Figure S1b**). Overall, we developed a computational tool which can predict the presence of both T9SS and gliding motility in members of the *Bacteroidetes* phylum, including mandatory and accessory protein components. Our prediction tool is available at: https://github.com/asamallab/T9GPred.

Classification of the secreted proteins into three C-terminal domain (CTD) types

To classify the proteins secreted via T9SS, we relied on the CTD of the compiled list of 102 secreted proteins (Methods). Earlier studies have reported the motif sequences for the CTD of the secreted proteins (31, 33), while some have additionally reported the motif classification (8, 29). We compiled a list of 18 CTD sequence motifs (Methods; **Table S6**) from the published literature and generated a motif similarity network (MSN) (**Figure S2**). From the MSN, we observed that the unclassified motifs clustered with the motifs classified as type A, whereas the motifs classified as type B are distinct (**Figure S2**). In addition, Kharade and McBride (11) reported a third type of CTD motif (type C) for the secreted proteins. Therefore, we accessed the HMM profiles from TIGRFAM (34) and NCBI protein

family models (https://www.ncbi.nlm.nih.gov/protfam) for all three types of CTD (type A, type B and type C) to classify the compiled list of 102 secreted proteins. We observed that the HMM profiles and the motif-based classification of the 102 secreted proteins yielded similar results with 68 proteins as type A, 18 proteins as type B and 1 protein as type C (**Figure 4**). From the classified CTD sequences, we generated HMM profiles (**Supplemental Information**). Additionally, we report CTD motif sequences specific to the classified proteins (**Table 1**). Importantly, these motif sequences are better at classifying the different types of CTD in comparison with the existing sequence motifs (**Figure 4**).

Computational pipeline to predict proteins secreted via T9SS

We present the first-ever computational pipeline to predict proteins secreted via T9SS across members of the *Bacteroidetes* phylum (**Figure 5**). The pipeline integrates several tools which predict important features in protein sequences. We used SignalP-5.0 (35) and Phobius (36) to detect the N-terminal Sec signal, SignalP-5.0 to detect N-terminal Tat signal, Phobius and TMHMM 2.0 (37) to detect the transmembrane domain (TM), and generated CTD HMM profiles to detect the CTD types in the protein sequences. We used 'OR rule' in the case where the feature is predicted by multiple tools. Based on the features of 102 experimentally identified proteins secreted via T9SS (**Table S3**), we designate a protein as secreted protein if: (i) it has Sec signal in N-terminal region; (ii) it has no Tat signal in N-terminal region; (iii) it has no TM domain; (iv) it has at least one of the three CTDs namely, type A, type B, or type C in its C-terminal region. We developed a computational tool implementing the above pipeline (**Figure 5**) to predict the secreted proteins from an input proteome. The source code of this computational tool is available at: https://github.com/asamallab/T9GPred. We used this tool to predict the secreted proteins for the 402 *Bacteroidetes* strains (**Table S5**) predicted to have T9SS. The predictions are available under the 'Secreted protein prediction' section of respective *Bacteroidetes* strain at https://cb.imsc.res.in/t9gpred/.

Comparative analysis of the secreted proteins from gliding and non-gliding *Bacteroidetes* strains

Using our computational tool, we predicted 327 *Bacteroidetes* strains to have gliding motility (gliding *Bacteroidetes*) and 75 *Bacteroidetes* strains to lack gliding motility (non-gliding *Bacteroidetes*) (Methods). Further, we also predicted the secreted proteins for both gliding and non-gliding *Bacteroidetes* using our computational tool. From our predictions, we noted that gliding *Bacteroidetes* secretes more proteins than the non-gliding *Bacteroidetes*. To check for the statistical significance of this difference, we first normalized the number of secreted proteins with the total number of proteins (coding sequences) in the respective *Bacteroidetes* strains. We observed that gliding *Bacteroidetes* significantly secrete more proteins than non-gliding *Bacteroidetes* ($p<0.001$; corrected using Bonferroni method; **Figure 6a**). We also observed that the genome of gliding *Bacteroidetes* is significantly larger than non-gliding *Bacteroidetes* ($p<0.01$; corrected using Bonferroni method; **Figure 6b**).

T9GPred web server

We have designed a Findable, Accessible, Interoperable and Reusable (FAIR) (38) compliant user-friendly web server, **T**ype **9** secretion system and **G**liding motility **Pred**iction (T9GPred) that compiles the predictions and the computational tool developed in this study. The web server is available at https://cb.imsc.res.in/t9gpred/. **Figure 7** shows the screenshots of the web interface of the T9GPred web server. T9GPred is an organized web server with simple navigation that enables easy access to users (**Figure 7a**).

Users can interactively browse for the 693 *Bacteroidetes* strains in the 'Browse' section of the navigation bar. Users can hover over the heatmap to see information on the name of the bacteria, the predicted protein components and the number of predicted protein sequences of the protein component in the proteome of the bacteria (**Figure 7b**). Users can

click on the name of the bacteria to see further details. The 'Bacteroidetes details' section gives the information on the organism name, genome identifier from PATRIC (34) or NCBI (https://www.ncbi.nlm.nih.gov/assembly/), isolation source of the bacteria, sequencing center information, predictions on T9SS, gliding motility and the number of secreted proteins, taxonomic information and proteome of the bacteria as a downloadable file (**Figure 7c**). Users can view the predicted protein components associated with T9SS on the 'T9SS prediction' page (**Figure 7d**) and the predicted protein components associated with gliding motility on the 'Gliding motility prediction' page (**Figure 7e**). For bacteria predicted to have T9SS, the predicted components can be visualized on the genome of the bacteria on the 'Gene cluster visualization' page (**Figure 7f**). Finally, the 'Secreted protein prediction' page lists the predicted secreted proteins and their annotations including the protein identifier from PATRIC (https://www.bv-brc.org/) or NCBI (https://www.ncbi.nlm.nih.gov/protein/), UniProt (39) identifier, AlphaFold DB (40, 41) identifier, type of evidence, type of CTD and the presence of Carbohydrate-Active enZYmes (CAZy) domain predicted using dbCAN2 tool (42) (**Figure 7g**).

Users can access the prediction tools in the 'Prediction' section of the navigation bar. The 'Prediction of T9SS and gliding motility' page contains the tool for the prediction of T9SS or gliding motility in the input proteome (**Figure 7h**). Users can upload the proteome in fasta file format or paste the sequence in the provided text box to run the prediction tool. The tool returns the predicted protein components along with the decision for the presence of T9SS or gliding motility (**Figure 7h**). The 'Prediction of protein secreted via T9SS' page allows users to predict whether the input protein can be secreted via T9SS (**Figure 7i**). Users can paste the sequence in the provided text box to run the prediction tool. The tool returns the prediction of different features including the classification of CTD types and mentions

whether the protein can be secreted via T9SS (**Figure 7i**). The standalone version of the prediction tool is available at our GitHub repository: https://github.com/asamallab/T9GPred.

DISCUSSION

T9SS is one of the least characterized secretion systems in Gram-negative bacteria (**Supplemental Information**; **Figure S3**). In this study, we leveraged the existing experimental evidence to develop the first comprehensive computational tool, T9GPred, which predicts the presence of T9SS, gliding motility and proteins secreted via T9SS from a bacterial proteome. In particular, we designated 6 key proteins (GldK, GldL, GldM, GldN, SprA and SprE) as mandatory for a functional T9SS. Using these 6 T9SS mandatory proteins, we predicted 402 of the 693 completely sequenced *Bacteroidetes* strains to have T9SS. Additionally, we also observed that our tool T9GPred predicted the presence of T9SS only in the *Bacteroidetes* phylum (**Supplemental Information**; **Table S8**) which is in line with the findings by McBride and colleagues (1, 3). Further, we also observed that the T9SS mandatory protein components are clustered in the bacterial genome (**Supplemental Information**; **Figure S4**) suggesting that GldK, GldL, GldM and GldN form an operon in members of the *Bacteroidetes* phylum having T9SS (12, 24).

In some *Bacteroidetes* strains, T9SS also aids in the movement of the species which is termed as gliding motility (1, 3). In addition to the 6 T9SS mandatory proteins, we designated 5 key proteins (GldB, GldD, GldH, GldJ and SprT) as mandatory for exhibiting gliding motility. Using these 11 gliding motility mandatory proteins, we predicted 327 of 402 *Bacteroidetes* strains with T9SS to also have gliding motility. Moreover, we observed that these 327 *Bacteroidetes* strains do not have flagellar motor or type IV pili (**Supplemental Information**). We also noted that there is no overlap between the protein components associated with gliding motility and flagellar motor or type IV pili (**Supplemental**

Information). Thus, our analysis suggests that gliding motility associated with T9SS is unique to the *Bacteroidetes* phylum.

The proteins secreted via T9SS are involved in multiple processes including the survival and pathogenicity of the bacteria (13, 28). We identified 3 types of C-terminal domain (CTD) signals (type A, type B or type C) by leveraging the reported signal sequences (8, 29, 31, 33) in conjunction with published evidence. We leveraged this CTD classification to build a computational pipeline which predicted the secreted proteins for the 402 *Bacteroidetes* strains having T9SS. A comparative analysis with the proteins secreted via other *Bacteroidetes*-specific secretion systems highlighted the uniqueness of the CTD signal in the proteins secreted via T9SS (**Supplemental Information**). We found that members of the *Bacteroidetes* phylum exhibiting gliding motility have significantly larger genome sizes and secrete more proteins in comparison with the members that do not have gliding motility (**Figure 6**). This suggests that members of the *Bacteroidetes* phylum exhibiting gliding motility have a larger genome probably encoding more specialized proteins to aid in gliding motility.

T9GPred has its fair share of limitations like any other computational tool. T9GPred does not mention the accuracy of the predictions as it lacks information on negative control. It relies on protein profiles trained on a small available set of experimentally characterized *Bacteroidetes* strains which limits its ability to exhaustively capture all variations. Moreover, T9GPred does not comment on the maturation of the secreted proteins as it does not include the information on the functional characterization of accessory protein components. The secreted protein prediction by T9GPred is limited by the choice of 3 CTD types considered in this study which is not indicative of all possible secreted proteins (8). Moreover, T9GPred does not include the structural conservation of the CTD (43) but considers only the protein sequence for the prediction of the secreted proteins. T9GPred predicts putative secreted

proteins but does not comment on the variation in the levels of secreted proteins in different environments.

Nevertheless, T9GPred aids in streamlining experiments by providing quick predictions. For pathogens, the predicted T9SS protein components and the secreted proteins could be targeted for drug discovery. For non-pathogens, T9GPred predicts secreted proteins that could be the putative subjects for genetic enhancements. T9GPred can also be used to predict the putative protein components in a draft genome since it relies on HMM profiles. T9GPred has the ability to identify a new uncharacterized bacterium as belonging to the *Bacteroidetes* phylum. In conclusion, we developed a computational tool T9GPred, that not only predicts T9SS but also predicts T9SS based gliding motility and the proteins secreted via T9SS. We have made the predictions and the tool available on our web server which is accessible at: https://cb.imsc.res.in/t9gpred/. The source code of the computational tool is available at: https://github.com/asamallab/T9GPred.

MATERIALS AND METHODS

Workflow to identify proteins associated with T9SS or gliding motility among members of the *Bacteroidetes* phylum

We mined PubMed (https://pubmed.ncbi.nlm.nih.gov/) to compile an exhaustive list of experimentally characterized proteins associated with T9SS or gliding motility in members of the *Bacteroidetes* phylum from published literature. We queried PubMed using the following keywords:

("type 9 secretion system"[Title/Abstract] OR "type 9 secretion systems"[Title/Abstract] OR "type IX secretion system"[Title/Abstract] OR "type IX secretion systems"[Title/Abstract] OR "type-9 secretion system"[Title/Abstract] OR "type-9 secretion systems"[Title/Abstract] OR "type-IX secretion system"[Title/Abstract] OR "type-IX secretion

systems”[Title/Abstract] OR “t9ss”[Title/Abstract] OR “T9SS”[Title/Abstract] OR “PoRSS”[Title/Abstract] OR “PoR secretion system”[Title/Abstract] OR “Type 9 secretion”[Title/Abstract] OR “Type IX secretion”[Title/Abstract])

and retrieved 107 published articles on 26 August 2020 (**Figure 1**). We noted that our PubMed query was unable to retrieve some previous studies focussing on gliding motility and the type 9 based secretion, as such studies were published prior to the use of T9SS nomenclature. Therefore, we manually compiled 22 additional published articles on gliding motility or type 9 based secretion (**Figure 1**). Thereafter, we excluded the articles which are computational studies or reviews; or which did not contain mutation-based experiments. Finally, we shortlisted 47 published articles from which we identified 23 proteins associated with T9SS (**Table S1**), 22 proteins associated with gliding motility (**Table S2**) and 102 proteins secreted via T9SS (secreted proteins) in members of the *Bacteroidetes* phylum (**Table S3**). **Figure 1** summarizes the workflow according to the PRISMA statement (44).

Compilation of completely sequenced *Bacteroidetes* strains

Our aim is to predict the presence of T9SS or gliding motility across the *Bacteroidetes* phylum. Thus, we considered *Bacteroidetes* strains for which the complete genome is available. We queried NCBI assembly (https://www.ncbi.nlm.nih.gov/assembly) using the following keywords:

(“Bacteroidetes”[Organism] or “Bacteroidia”[Organism] or Bacteroidetes[All Fields]) and (bacteria[filter] and “latest refseq”[filter] and “complete genome”[filter] and all[filter] not anomalous[filter])

and identified 693 completely sequenced *Bacteroidetes* strains on 21 December 2020 (**Table S5**). For these 693 *Bacteroidetes* strains, we accessed the corresponding proteome from PATRIC (45) (https://www.patricbrc.org/) or NCBI assembly

(https://www.ncbi.nlm.nih.gov/assembly). Note that if the proteome for a bacterium was available in both PATRIC and NCBI assembly, we preferred the proteome from PATRIC.

Bioinformatics analysis to identify and predict T9SS or gliding motility

Among the 693 completely sequenced *Bacteroidetes* strains, we identified 43 *Bacteroidetes* strains to have experimentally or computationally characterized proteins associated with T9SS or gliding motility from published literature (**Table S7**; **Supplemental Information**). Further, we used these 43 proteomes to generate Hidden Markov Model (HMM) profiles for the identified protein components associated with T9SS or gliding motility. The detailed workflow to generate HMM profiles is documented in the **Supplemental Information**. Subsequently, we relied solely on the experimental evidence from 30 *Bacteroidetes* strains to infer the mandatory protein components for T9SS and gliding motility (**Table S4**). We collated the HMM profiles of the designated mandatory components and scripted a python program that checks for the presence of T9SS or gliding motility across the 693 completely sequenced *Bacteroidetes* strains.

Computational analysis of secreted proteins

We collected the sequences of the identified 102 secreted proteins (**Table S3**) from NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/). We considered the last 120 amino acids of each protein sequence as their C-terminal domain (CTD) (8). Further, we used ClustalW (46) with default parameters to align the CTD sequences of the 102 secreted proteins. Thereafter, we clustered the aligned sequences using MEGA X (47) with the maximum-likelihood method and default parameters.

We relied on HMM profile based prediction and motif analysis to classify the 102 CTD sequences. We accessed the HMM profiles for CTD types from TIGRFAM (34) and NCBI protein family models (https://www.ncbi.nlm.nih.gov/protfam) and compiled 18 CTD

sequence motifs of secreted proteins from published literature (**Table S6**). To find the similarity between the motifs, we created a motif similarity network (MSN) using Tomtom (48) (https://meme-suite.org/meme/tools/tomtom) by setting the overlap >= 3 and score >= 2 (49) and visualized the MSN using Cytoscape (50) (**Figure S2**). Further, we determined the presence of the motifs in the CTD of the secreted proteins using Find Individual Motif Occurrence (FIMO) (https://meme-suite.org/meme/tools/fimo) tool (51) with default parameters. We classified the CTD types of secreted proteins based on the HMM prediction and substantiated with motif based classification. Using the classified CTD sequences, we created the corresponding HMM profiles (**Supplemental Information**) and generated motifs using Multiple Expectation Maximization for Motif Elicitation (MEME) (52) (https://meme-suite.org/meme/tools/meme) tool in the discriminative mode of motif discovery.

Web interface and web server management system

We created a user-friendly web server **T**ype **9** secretion system and **G**liding motility **Pred**iction (T9GPred) to compile and share the predictions and the computational tool generated in this study. T9GPred compiles the proteins associated with T9SS or gliding motility in the 693 completely sequenced *Bacteroidetes* strains. For the *Bacteroidetes* strains predicted to have T9SS, T9GPred lists the proteins predicted to be secreted via T9SS. Moreover, T9GPred contains prediction tool to check for presence of T9SS or gliding motility, and secreted proteins. T9GPred is openly available at https://cb.imsc.res.in/t9gpred/.

To create the web server, we used MariaDB (https://mariadb.org/) to store the compiled information and Structured Query Language (SQL) to retrieve information. We used PHP (https://www.php.net/) with custom HTML, CSS, jQuery (https://jquery.com/), Bootstrap 4 (https://getbootstrap.com/docs/4.0/), and Plotly JavaScript (https://plotly.com/javascript/) to create the web interface of T9GPred. T9GPred web server

is hosted on an Apache (https://httpd.apache.org/) server running on Debian 9.4 Linux Operating System.

Data availability

The predictions and the computational tool are accessible via the web server, T9GPred available at: https://cb.imsc.res.in/t9gpred/. The standalone version of the developed computational tool and the codes to generate different plots are available in our GitHub repository: https://github.com/asamallab/T9GPred.

AUTHOR CONTRIBUTIONS

Ajaya Kumar Sahoo: Conceptualization, Data Compilation, Data Curation, Formal Analysis, Software, Visualization, Writing; **R. P. Vivek-Ananth:** Conceptualization, Formal Analysis, Software, Visualization, Writing; **Nikhil Chivukula:** Conceptualization, Formal Analysis, Software, Visualization, Writing; **Shri Vishalini Rajaram:** Conceptualization, Data Compilation, Data Curation, Formal Analysis; **Karthikeyan Mohanraj:** Software, Visualization; **Devanshi Khare:** Formal Analysis; **Celin Acharya:** Conceptualization, Formal Analysis, Writing; **Areejit Samal:** Conceptualization, Supervision, Formal Analysis, Software, Writing.

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FIGURE CAPTIONS

Figure 1: Workflow to identify published research articles containing experimental evidence on the proteins associated with T9SS or gliding motility or proteins secreted via T9SS. This workflow is presented according to the PRISMA statement (44).

Figure 2: Occurrence of 28 identified protein components associated with T9SS or gliding motility checked across 30 experimentally characterized *Bacteroidetes* strains using the generated HMM profiles. The presence of T9SS mandatory components are denoted by light brown colored box, gliding motility mandatory components are denoted by cyan colored box, and T9SS or gliding motility accessory components are denoted by dark brown colored box. The experimental evidence (presence, absence or no evidence) for T9SS or gliding motility is denoted alongside each *Bacteroidetes* strain.

Figure 3: Schematic diagram encapsulating the information on localization of 28 protein components on the bacterial membrane based on published literature.

Figure 4: Identification and classification of the C-terminal domain (CTD;last 120 aa) of 102 experimentally characterized secreted proteins into three CTD types (type A, type B or type C) based on HMM profiles and sequence motifs from published literature. The motifs from Seers *et al.* (33) and Veith *et al.* (31) are classified as type A CTD based on the motif similarity network (**Figure S2**). Based on the classification of the secreted proteins, we generated 4 motifs for type A and 3 motifs for type B. The CTD of the secreted proteins are clustered using ClustalW (46) and the colored dots represent the presence of the CTD types. The figure was generated using Evolview (53) web server (https://www.evolgenius.info/evolview/).

Figure 5: The computational pipeline to predict proteins secreted via T9SS. Computational tools used in this pipeline to predict features in the input protein sequence are mentioned in the top right panel in the figure.

Figure 6: Comparative analysis of the secreted proteins and genome size between gliding and non-gliding *Bacteroidetes* strains predicted in this study. **(a)** Comparison of the predicted secreted proteins normalized with the total number of coding sequences of respective bacteria (ratio) between gliding and non-gliding *Bacteroidetes* strains (significantly different at $p<0.001$). **(b)** Comparison of the genome sizes (in kb) between gliding and non-gliding *Bacteroidetes* strains (significantly different at $p<0.01$). In each case, the significant difference between two samples is calculated using Bonferroni correction in rstatix package (https://cran.r-project.org/web/packages/rstatix/index.html) available in R version 4.2.1. Both plots are made using the ggplot2 (54) package in R version 4.2.1.

Figure 7: Snapshots of the web interface of the T9GPred web server. (a) 'Home' page showing navigation bar. (b) 'Browse' option enables users to explore the details and predictions for 693 completely sequenced *Bacteroidetes* strains considered in this study. (c) The detailed information page for selected *Bacteroidetes* strain. (d) The 'T9SS prediction' page lists the predicted protein components associated with T9SS and visualizes the predicted components. (e) The 'Gliding motility prediction' page lists the predicted protein components associated with gliding motility and visualizes the predicted components. (f) The 'Gene cluster visualization' page provides a visualization of the location of the predicted protein components in the genome of the selected *Bacteroidetes strain*. (g) The 'Secreted protein prediction' page tabulates the proteins predicted to be secreted via T9SS of the selected *Bacteroidetes* strain. (h) The 'Prediction of T9SS and gliding motility' page allows users to submit the proteome of a bacterium and provides the prediction on the T9SS and gliding motility in a tabular format. (i) The 'Prediction of protein secreted via T9SS' page

allows users to submit a protein sequence and provides the prediction of the features in a tabular format.

TABLES

Table 1: The table lists four type A and three type B CTD motifs generated in this study. We used the 'discriminative mode' in the MEME tool (https://meme-suite.org/meme/tools/meme) to generate the motifs from the input list of experimentally characterized secreted proteins. For each motif, we also provide the e-value given by the MEME tool.

Motif Identifier	**Motif**	**E-value**
Type A_1	KIYPNPVSEILNIALQE[GN]LQL[EQ]KVNFYNTLGQLIKTTNHSE[IT]N[VI]SSFAKG	7.3e-454
Type A_2	VF[GR][ND]V[TN][QK]SNCALNVP[AT]GT[EQ]A[AV]YQAAAVW[KR][DN]FSPISG[SN]LLSNHSFAIES[NA]L	2.6e-398
Type A_3	VK[VI]YPN[PQ]GKGT[LK][TN]I[SI]	1.3e-158
Type A_4	[LV]SNLA[KS]G[VI]Y[IFL][VL]K[IV]XX	7.4e-115
Type B_1	F[TS]PNGDGYNDT[WF]Y[IP]E[NG][IL]EXYP	2.5e-091
Type B_2	ELP[SA]G[DT]Y[WY][YF][VI][LVI]K[YF]N[DE]N[KN][NT]XK	6.4e-067
Type B_3	[VI]EI[FY][DN]R[YWF]G[KRV]L[IV][KY]ELDG[NYG]DNG[WD]	6.1e-070

Supplemental Information

Supplemental Information consists of Supplemental Text, Supplemental Figures S1-S5 and Supplemental Tables S1-S10.

Supplemental Figures

Figure S1: (a) Distribution of the number of proteins associated with T9SS in each of 402 *Bacteroidetes* strains predicted to have T9SS. The mean and median of the distribution are 19.30 and 19, respectively. **(b)** Distribution of the number of proteins associated with T9SS or gliding motility in each of 327 *Bacteroidetes* strains predicted to exhibit gliding motility. The mean and median of the displayed distribution are 21.98 and 22, respectively.

Figure S2: Motif similarity network (MSN) of the 18 compiled CTD motifs generated using Tomtom software by setting the overlap >= 3 and score >= 2. We visualized the MSN using Cytoscape, where each node represents the motifs and the edges represent the score computed using Tomtom.

Figure S3: Cumulative estimate of published research articles for the nine different secretion systems (T1SS-T9SS) in bacteria. We have not considered T10SS or T11SS in this estimate as these two systems are very recently discovered.

Figure S4: Gene cluster visualization of the proteins associated with T9SS or gliding motility in the genome of *Flavobacterium johnsoniae* UW101 and *Porphyromonas gingivalis* ATCC 33277. The operons consisting of GldK (1) GldL (2) GldM (3) GldN (4) are shown in the enlarged view. The figure was generated using CGView package.

Figure S5: Flowchart describing the workflow to create the HMM profile from protein sequences.

Supplemental Tables

Table S1: The table gives the compiled list of 23 proteins with experimental evidence of being associated with T9SS in members of the *Bacteroidetes* phylum. For each protein, the table gives the GenBank protein accession from respective *Bacteroidetes* strain, type of experimental evidence, and PubMed identifier of literature reference(s).

Table S2: The table gives the compiled list of 22 proteins with experimental evidence of being associated with gliding motility in members of the *Bacteroidetes* phylum. For each protein, the table gives the GenBank protein accession from respective *Bacteroidetes* strain, type of experimental evidence, and PubMed identifier of literature reference(s).

Table S3: The table gives the compiled list of 102 proteins with published experimental evidence of being secreted via T9SS in members of *Bacteroidetes* phylum. For each protein, the table gives the GenBank protein accession, *Bacteroidetes* strain in which the protein was identified, type of experimental evidence, and PubMed identifier of literature reference(s). Moreover, the table gives for each protein sequence, the Sec signal prediction from SignalP and Phobius, Tat signal prediction from SignalP, and the Transmembrane domain prediction from Phobius and TMHMM.

Table S4: The table gives the list of 30 *Bacteroidetes* strains with experimental evidence on the presence or absence of T9SS or gliding motility. For each *Bacteroidetes* strain, the table also gives PubMed identifier of literature reference(s).

Table S5: The table gives the compiled list of 693 completely sequenced *Bacteroidetes* strains considered for this study. For each *Bacteroidetes* strain, the table provides taxonomic information, isolation source, sequencing center information, prediction of T9SS, gliding motility and the number of proteins predicted to be secreted via T9SS.

Table S6: The table gives the list of 18 sequence motifs for the C-terminal domain (CTD) of the proteins secreted via T9SS. For each motif, the table gives the motif identifier which is based on the author name, type of CTD motif and the PubMed identifier of the literature reference.

Table S7: The table gives the compiled list of 43 *Bacteroidetes* strains considered to generate Hidden Markov Model (HMM) profiles for 28 proteins associated with T9SS or gliding motility. For each *Bacteroidetes* strain, the table provides the genome identifier, true positive hit(s) of the 28 proteins, type of evidence of protein characterization and the PubMed identifier of literature reference(s).

Table S8: The table gives the list of 34,369 completely sequenced bacteria from NCBI assembly. For each bacterium, the table gives the phylum information and the prediction of T9SS using T9GPred. Note that the phylum *Bacteroidetes* and *Bacteroidota* are synonyms according to NCBI Taxonomy 2023 (https://ncbiinsights.ncbi.nlm.nih.gov/2022/11/14/prokaryotic-phylum-name-changes/).

Table S9: The table gives the PubMed query for the each of the nine different secretion systems (T1SS-T9SS) in bacteria and the respective number of articles retrieved. The PubMed search was last performed on 10 January 2021.

Table S10: The table gives the true positive hit for the type C C-terminal domain (CTD) generated from analyzing 693 completely sequenced *Bacteroidetes* strains.

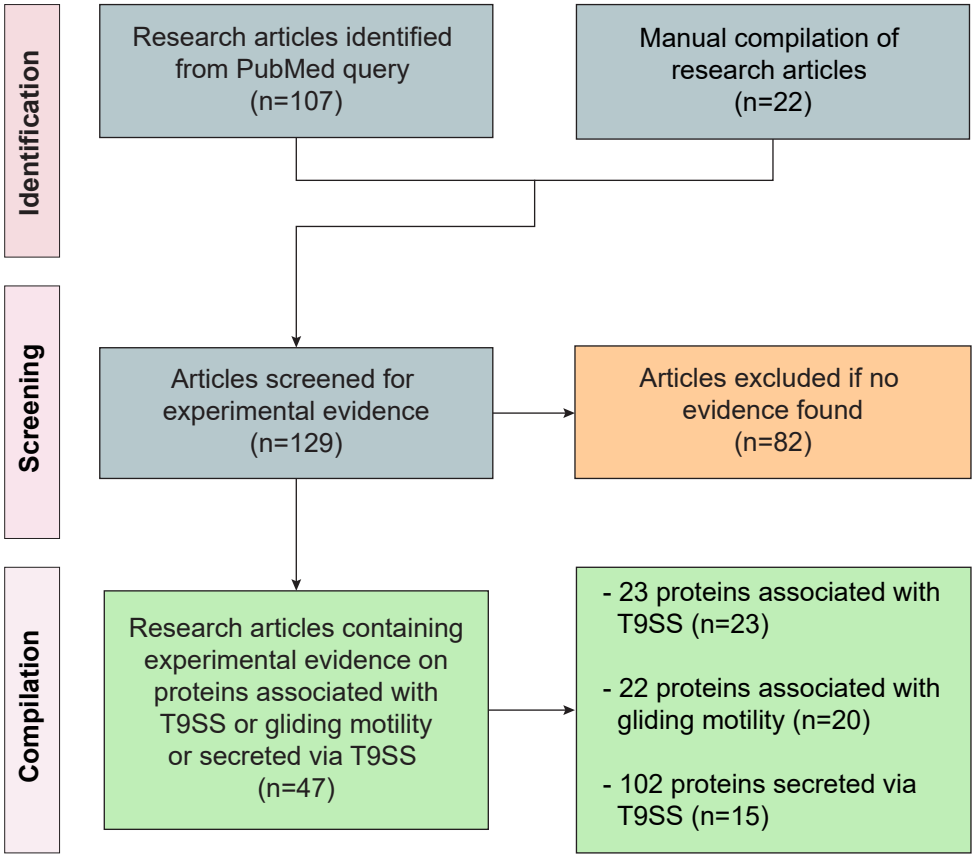
Identification
Research articles identified from PubMed query (n=107)
Manual compilation of research articles (n=22)
Screening
Articles screened for experimental evidence (n=129)
Articles excluded if no evidence found (n=82)
Compilation
Research articles containing experimental evidence on proteins associated with T9SS or gliding motility or secreted via T9SS (n=47)
- 23 proteins associated with T9SS (n=23)
- 22 proteins associated with gliding motility (n=20)
- 102 proteins secreted via T9SS (n=15)

Figure 1

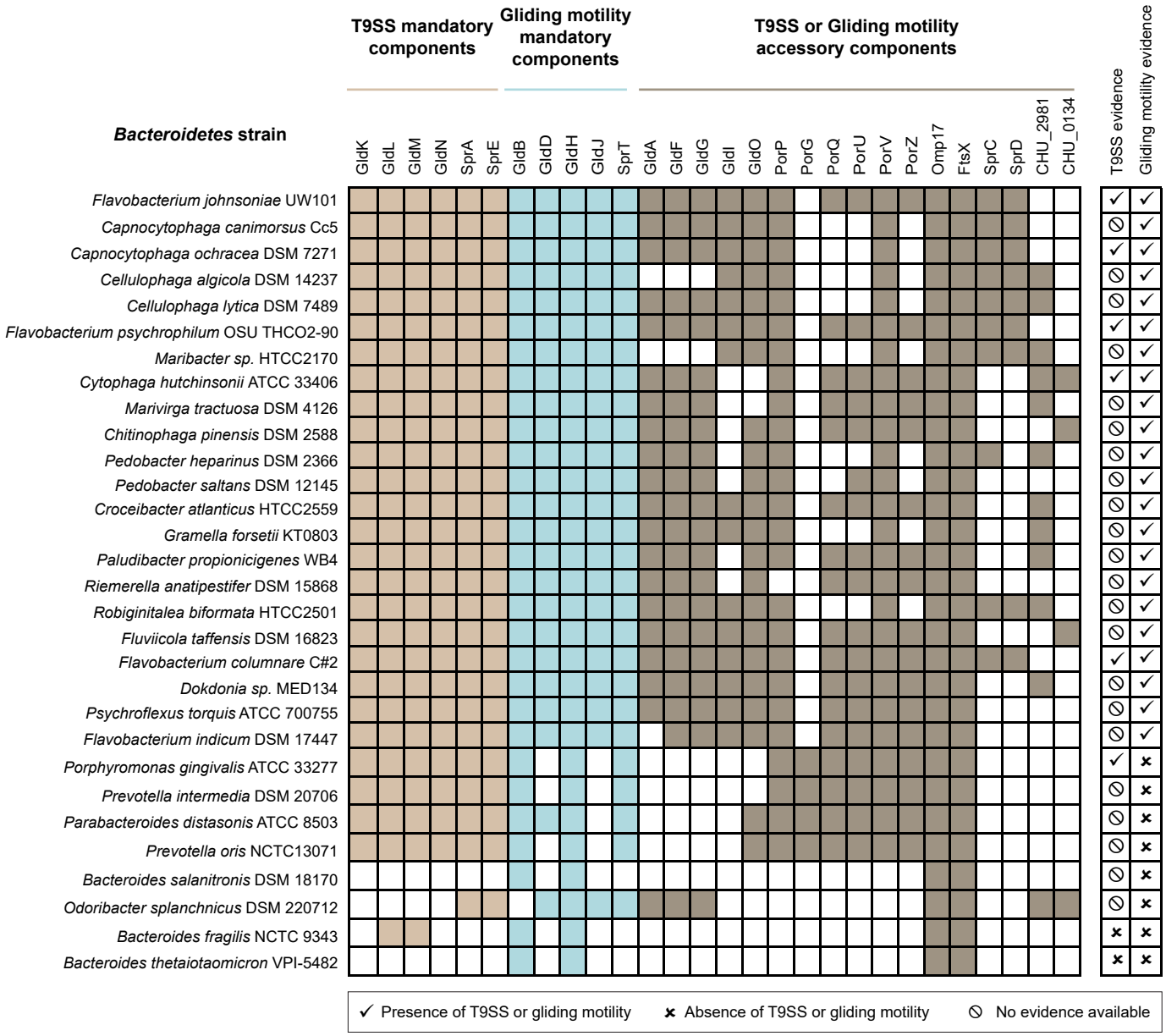
T9SS mandatory components
Gliding motility mandatory components
T9SS or Gliding motility accessory components
Bacteroidetes strain
GldK
GldL
GldM
GldN
SprA
SprE
GldB
GldD
GldH
GldJ
SprT
GldA
GldF
GldG
GldI
GldO
PorP
PorG
PorQ
PorU
PorV
PorZ
Omp17
FtsX
SprC
SprD
CHU_2981
CHU_0134
T9SS evidence
Gliding motility evidence
Flavobacterium johnsoniae UW101
Capnocytophaga canimorsus Cc5
Capnocytophaga ochracea DSM 7271
Cellulophaga algicola DSM 14237
Cellulophaga lytica DSM 7489
Flavobacterium psychrophilum OSU THCO2-90
Maribacter sp. HTCC2170
Cytophaga hutchinsonii ATCC 33406
Marivirga tractuosa DSM 4126
Chitinophaga pinensis DSM 2588
Pedobacter heparinus DSM 2366
Pedobacter saltans DSM 12145
Croceibacter atlanticus HTCC2559
Gramella forsetii KT0803
Paludibacter propionicigenes WB4
Riemerella anatipestifer DSM 15868
Robiginitalea biformata HTCC2501
Fluviicola taffensis DSM 16823
Flavobacterium columnare C#2
Dokdonia sp. MED134
Psychroflexus torquis ATCC 700755
Flavobacterium indicum DSM 17447
Porphyromonas gingivalis ATCC 33277
Prevotella intermedia DSM 20706
Parabacteroides distasonis ATCC 8503
Prevotella oris NCTC13071
Bacteroides salanitronis DSM 18170
Odoribacter splanchnicus DSM 220712
Bacteroides fragilis NCTC 9343
Bacteroides thetaiotaomicron VPI-5482
✓ Presence of T9SS or gliding motility
✗ Absence of T9SS or gliding motility
⊘ No evidence available

Figure 2

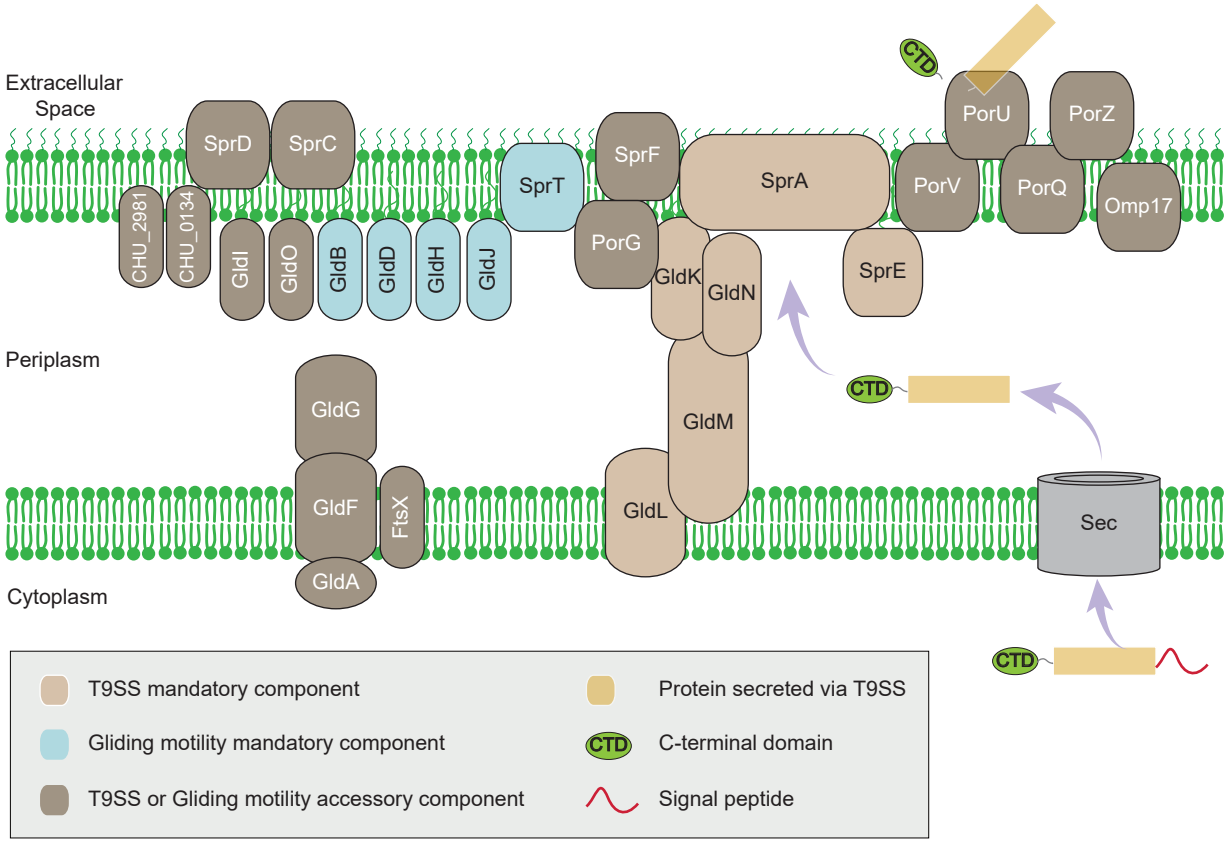
Extracellular Space
Periplasm
Cytoplasm
SprD
SprC
CHU_2981
CHU_0134
GldI
GldO
GldB
GldD
GldH
GldJ
SprT
SprF
PorG
SprA
GldK
GldN
GldM
GldL
SprE
PorV
PorU
PorQ
PorZ
Omp17
CTD
GldG
GldF
FtsX
GldA
Sec
T9SS mandatory component
Gliding motility mandatory component
T9SS or Gliding motility accessory component
Protein secreted via T9SS
C-terminal domain
Signal peptide

Figure 3

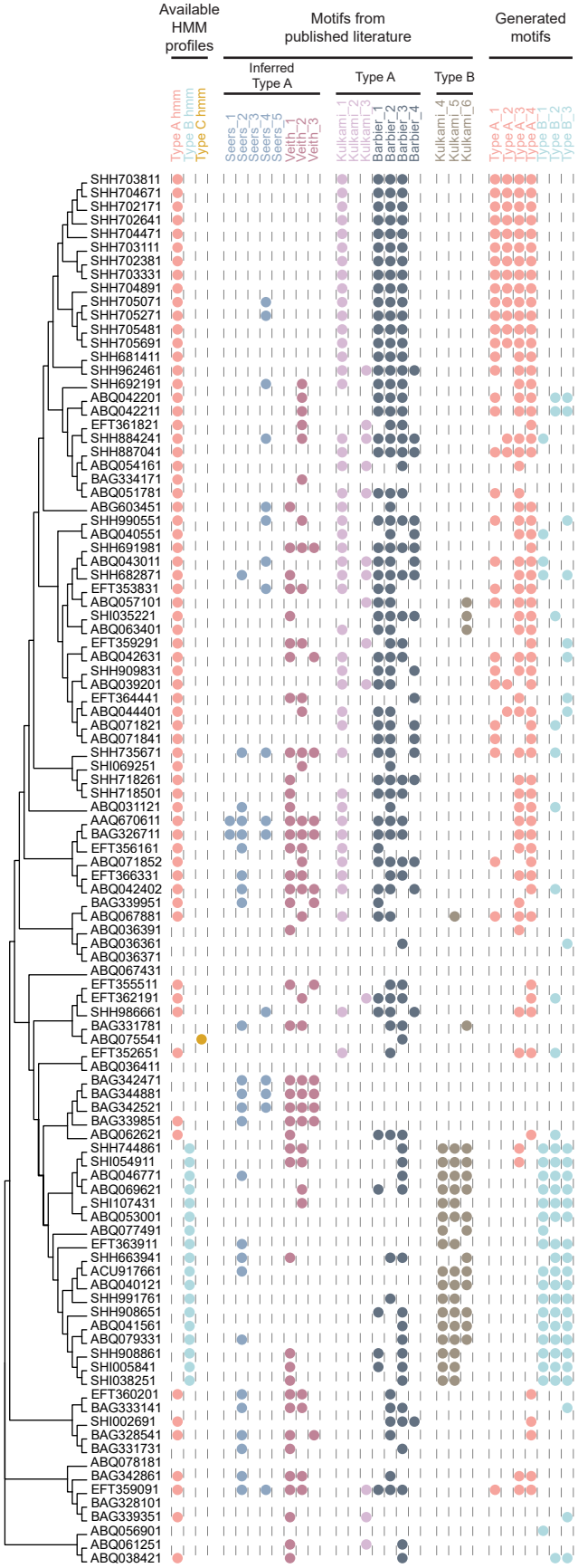
Available HMM profiles
Motifs from published literature
Generated motifs
Inferred Type A
Type A
Type B
Type A hmm
Type B hmm
Type C hmm
Seers_1
Seers_2
Seers_3
Seers_4
Seers_5
Veith_1
Veith_2
Veith_3
Kulkami_1
Kulkami_2
Kulkami_3
Barbier_1
Barbier_2
Barbier_3
Barbier_4
Kulkami_4
Kulkami_5
Kulkami_6
Type A_1
Type A_2
Type A_3
Type A_4
Type B_1
Type B_2
Type B_3
SHH703811
SHH704671
SHH702171
SHH702641
SHH704471
SHH703111
SHH702381
SHH703331
SHH704891
SHH705071
SHH705271
SHH705481
SHH705691
SHH681411
SHH962461
SHH692191
ABQ042201
ABQ042211
EFT361821
SHH884241
SHH887041
ABQ054161
BAG334171
ABQ051781
ABG603451
SHH990551
ABQ040551
SHH691981
ABQ043011
SHH682871
EFT353831
ABQ057101
SHI035221
ABQ063401
EFT359291
ABQ042631
SHH909831
ABQ039201
EFT364441
ABQ044401
ABQ071821
ABQ071841
SHH735671
SHI069251
SHH718261
SHH718501
ABQ031121
AAQ670611
BAG326711
EFT356161
ABQ071852
EFT366331
ABQ042402
BAG339951
ABQ067881
ABQ036391
ABQ036361
ABQ036371
ABQ067431
EFT355511
EFT362191
SHH986661
BAG331781
ABQ075541
EFT352651
ABQ036411
BAG342471
BAG344881
BAG342521
BAG339851
ABQ062621
SHH744861
SHI054911
ABQ046771
ABQ069621
SHI107431
ABQ053001
ABQ077491
EFT363911
SHH663941
ACU917661
ABQ040121
SHH991761
SHH908651
ABQ041561
ABQ079331
SHH908861
SHI005841
SHI038251
EFT360201
BAG333141
SHI002691
BAG328541
BAG331731
ABQ078181
BAG342861
EFT359091
BAG328101
BAG339351
ABQ056901
ABQ061251
ABQ038421

Figure 4

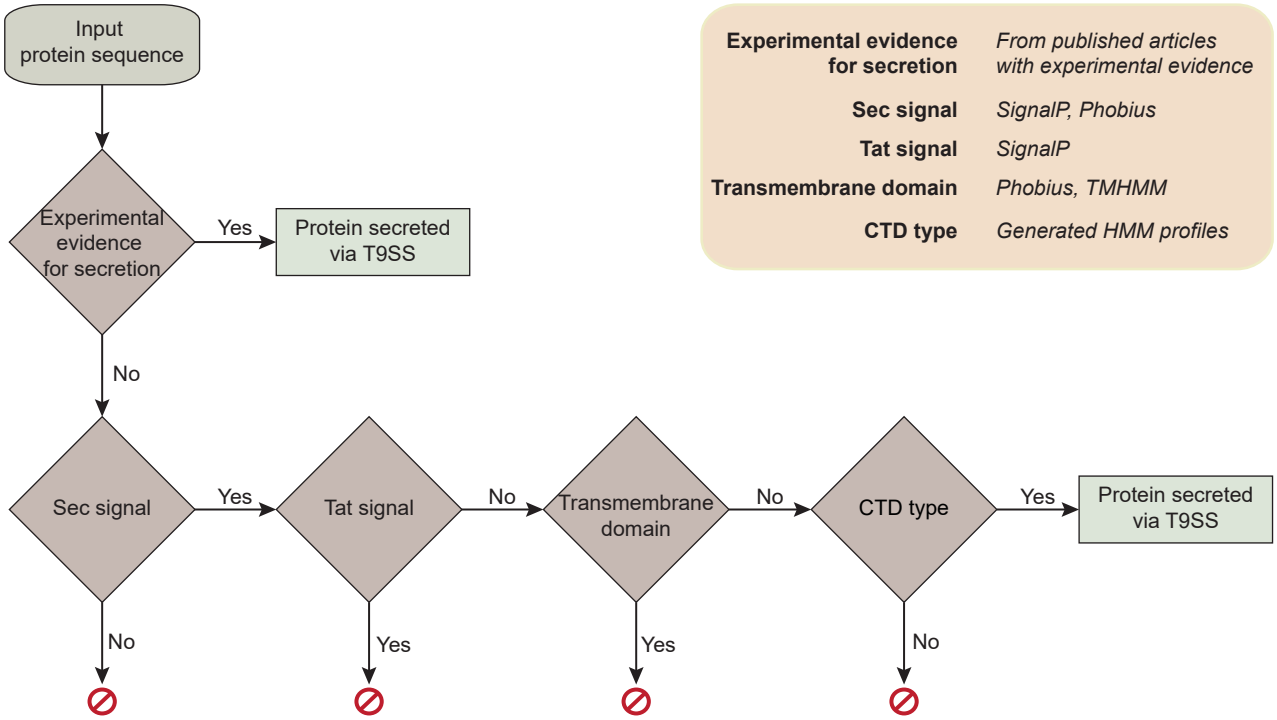
Input protein sequence
Experimental evidence for secretion
Yes
Protein secreted via T9SS
No
Sec signal
Yes
Tat signal
No
Transmembrane domain
No
CTD type
Yes
Protein secreted via T9SS
No
Yes
Yes
No
Experimental evidence for secretion
From published articles with experimental evidence
Sec signal
SignalP, Phobius
Tat signal
SignalP
Transmembrane domain
Phobius, TMHMM
CTD type
Generated HMM profiles

Figure 5

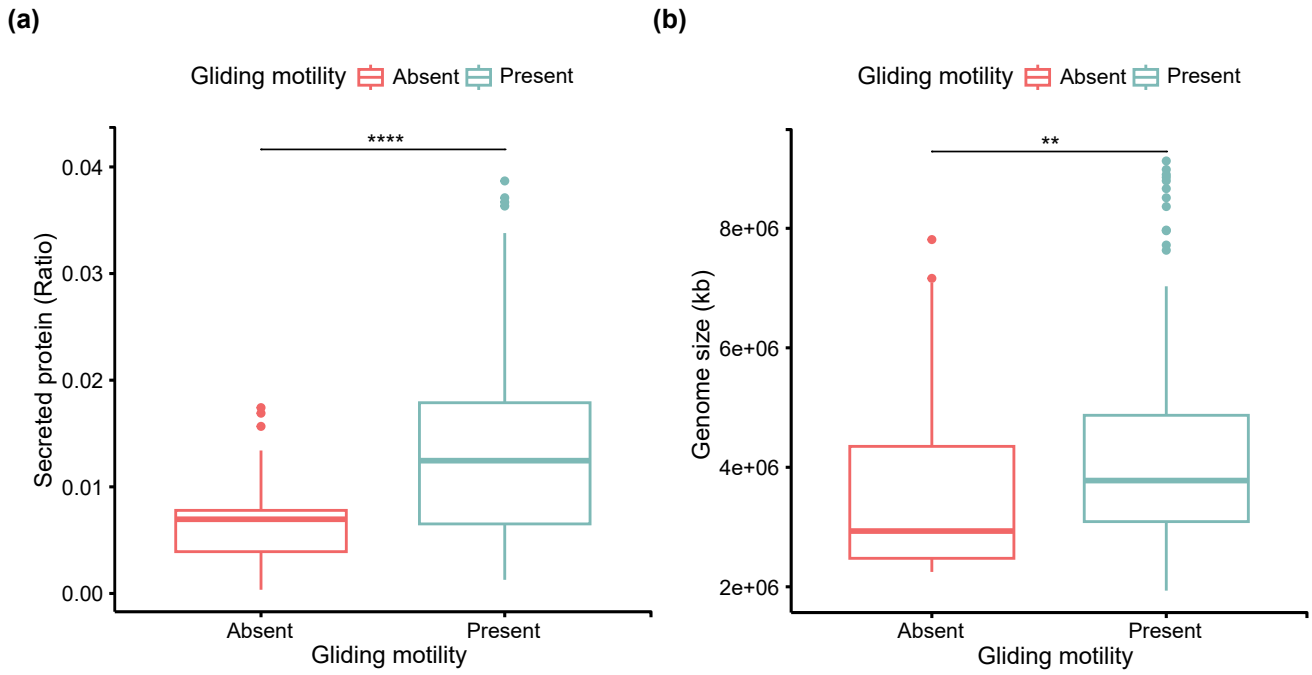
(a)
Gliding motility
Absent
Present
****
Secreted protein (Ratio)
0.04
0.03
0.02
0.01
0.00
Absent
Present
Gliding motility
(b)
Gliding motility
Absent
Present
**
Genome size (kb)
8e+06
6e+06
4e+06
2e+06
Absent
Present
Gliding motility

Figure 6

(a)
T9GPred
Type 9 secretion system and Gliding motility Prediction
Home
Browse
Prediction
Acknowledgement
Help
T9SS
Gliding motility
N-terminal signal
C-terminal signal
Secreted protein prediction
(b)
Browse Bacteroidetes
T9SS mandatory protein
Gliding motility mandatory protein
T9SS or gliding motility accessory protein
Protein secreted via T9SS
Not predicted
(c)
Bacteroidetes: Flavobacterium johnsoniae UW101
Bacteroidetes details
T9SS prediction
Gliding motility prediction
Gene cluster visualization
Secreted protein prediction
Details
Organism
Flavobacterium johnsoniae UW101
Genome Identifier
PATRIC:376686.10
Isolation source
Soil
Sequencing center
DOE Joint Genome Institute
Prediction
T9SS
Present
Gliding motility
Present
Secreted protein
51
Taxonomy
Kingdom
Bacteria
Phylum
Bacteroidetes
Class
Flavobacteriia
Order
Flavobacteriales
Family
Flavobacteriaceae
Genus
Flavobacterium
Download proteome:
fasta
(d)
Bacteroidetes: Flavobacterium johnsoniae UW101
Bacteroidetes details
T9SS Prediction
Gliding motility prediction
Gene cluster visualization
Secreted protein prediction
Proteins associated with T9SS
GldA
fig|376686.10.peg.1562|Fjoh_1516
GldB
fig|376686.10.peg.1841|Fjoh_1793
GldD
fig|376686.10.peg.1585|Fjoh_1540
GldF
fig|376686.10.peg.2810|Fjoh_2722
GldG
fig|376686.10.peg.2809|Fjoh_2721
GldH
fig|376686.10.peg.916|Fjoh_0890
T9SS components
Prediction: T9SS is present
(e)
Bacteroidetes: Flavobacterium johnsoniae UW101
Bacteroidetes details
T9SS Prediction
Gliding motility prediction
Gene cluster visualization
Secreted protein prediction
Proteins associated with gliding motility
FtsX
fig|376686.10.peg.585|Fjoh_0567
GldA
fig|376686.10.peg.1562|Fjoh_1516
GldB
fig|376686.10.peg.1841|Fjoh_1793
GldD
fig|376686.10.peg.1585|Fjoh_1540
GldF
fig|376686.10.peg.2810|Fjoh_2722
GldG
fig|376686.10.peg.2809|Fjoh_2721
Gliding motility components
Prediction: Gliding motility is present
(f)
Bacteroidetes: Flavobacterium johnsoniae UW101
Bacteroidetes details
T9SS Prediction
Gliding motility prediction
Gene cluster visualization
Secreted protein prediction
Accession: NC_009441
Length: 6096872 bp
T9SS mandatory components
Gliding motility mandatory components
T9SS/Gliding motility accessory componen
Flavobacterium johnsoniae
UW101
Expand -
Expand +
Full view
Rotate -
Rotate +
(g)
Bacteroidetes: Flavobacterium johnsoniae UW101
Bacteroidetes details
T9SS Prediction
Gliding motility prediction
Gene cluster visualization
Secreted protein prediction
Serial Number
Protein Identifier (PATRIC / NCBI Protein)
UniProt Identifier
AlphaFoldDB Identifier
Type of Evidence
CTD Type
CAZy Domain
1
fig|376686.10.peg.871|Fjoh_0848
Computational
TypeA
2
fig|376686.10.peg.5121|Fjoh_4948
Computational
TypeA
3
fig|376686.10.peg.2406|Fjoh_2338
Computational
TypeA
4
fig|376686.10.peg.4590|Fjoh_4436
Computational
TypeA
GH27|CBM13
5
fig|376686.10.peg.3413|Fjoh_3296
Computational
TypeA
6
fig|376686.10.peg.2407|Fjoh_2339
Computational
TypeA
(h)
Upload fasta file
(See Example.fasta)
Choose File
Example.fasta
Note: Provide only one input sequence at a time. The user input sequence will not be saved locally or used for any other purpose.
Submit
Example.fasta
Protein
Hits
GldN
ABQ04889.2
GldN
ABQ04888.1
SprD
ABQ04013.1
GldF
ABQ05744.1
GldM
ABQ04887.1
Omp17
ABQ04721.1
T9SS prediction: T9SS is present
Gliding motility prediction: Gliding motility is present
(i)
(See Example.fasta)
Note: Provide only one input sequence at a time. The user input sequence will not be saved locally or used for any other purpose.
Submit
Type 9 secreted protein prediction
ABQ03842.1 is a protein secreted via T9SS
Features
Prediction
Signal peptide
Present
TAT signal
Absent
Transmembrane domain
Absent
Type of CTD
TypeA

Figure 7