Type-IVC Secretion System: A Novel Subclass of Type IV Secretion System (T4SS) Common Existing in Gram-Positive Genus *Streptococcus*

Wen Zhang1*, Chengbo Rong2*, Chen Chen1*, George F. Gao1,2,3*

1 National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention/State Key Laboratory for Infectious Disease Prevention and Control, Beijing, China, 2CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Science, Beijing, China, 3 Beijing Institutes of Life Science, Chinese Academy of Sciences, Beijing, China

Abstract

A growing number of pathogens are being found to possess specialized secretion systems which they use in various ways to subvert host defenses [1]. Type IV secretion system (T4SS) is one of versatile secretion systems essential for the virulence and even survival of some bacteria species, and they enable the secretion of protein and DNA substrates across the cell envelope. T4SS was once believed to be present only in Gram-negative bacteria. In this study, we present evidence of a new subclass of T4SS, Type-IVC secretion system and indicate its common existence in the Gram-positive bacterial genus *Streptococcus*. We further identified that VirB1, VirB4, VirB6 and VirD4 are the minimal key components of this system. Using genome comparisons and evolutionary relationship analysis, we proposed that Type-IVC secretion system is movable via transposon factors and mediates the conjugative transfer of DNA, enhances bacterial pathogenicity, and could cause large-scale outbreaks of infections in humans.

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* E-mail: gaof@im.ac.cn (GFG); chenchen@icdc.cn (CC)

† These authors contributed equally to this work.

**Introduction**

A growing number of pathogens are being found to possess specialized secretion systems which they use in various ways to subvert host defenses [1]. Type IV secretion system (T4SS) is one of versatile secretion systems essential for the virulence and even survival of some bacteria species, and they enable the secretion of protein and DNA substrates across the cell envelope [2,3,4,5].

Based on a number of characteristics, including, the organization of genetic determinants, shared homologies and evolutionary relationships, T4SSs have been divided into several subgroups: Type-IVA, Type-IVB systems and other T4SSs [6,7]. The *Agrobacterium tumefaciens* Vir system is considered to be the paradigm of Type-IVA, which consists of 12 components, VirB1–VirB11 and VirD4 [7]. VirB1, with its lytic transglycosylase subunits, can create holes in the cell wall to enable the movement of T4SS [3,8]. VirB2 and VirB3 are pilus components [3,9,10]. Three cytoplasmic ATPases, VirB4, VirB11, and VirD4, provide energy for substrate secretion and assist in the assembly of the T4SS [3]. VirD4 is referred to as a coupling protein which recruit the substrates to the T4SS for translocation [11,12]. VirB6 and VirB8 are polytopic inner membrane proteins essential for substrate secretion through the inner membrane in Gram-negative bacteria [3,13], while VirB7–VirB9–VirB10 forms a stable core complex that spans the cell membrane [3,14]. The type-IVB secretion system was initially found in *Legionella pneumophila* and is composed of 25 genes on two separate regions. Region I contains seven genes (*icmV, W and X, and dotA, B, C and D*), and Region II contain the other 18 genes (*icmT, S, R, Q, P, O, N, M, L, K, E, G, C, D, j, B, F and H*) [15].

The majority of these genes were also found in the genome sequences of *Coxiella burnetti* [15,16]. More recently, a novel lineage of T4SSs classified as “others” have been identified on the genomic island ICE*Hin1056* of *Hymenobacterium influenzae* [17,18].

T4SS was once believed to be present only in Gram-negative bacteria but has been found in Gram-positive organisms as well. Our previous work support that in Gram-positive species of *Streptococcus suis* (*S. suis*), a GI-type T4SS-like system was identified in a new pathogenicity island (PAI) with a length of 89 kb [18], which was further proven to be a new subgroup of T4SS in this study. *S. suis*, a Gram-positive species of *Streptococcus* found in pigs, has recently caused a rash of human infections in China and gained public attention [19,20,21,22]. In these epidemic *S. suis* isolates related to two recent large-scale outbreaks of human infection in China, we identified this new 89 kb PAI with GI-type T4SS in pigs [18]. Our works also showed that this 89K PAI can spontaneously excise to form an extrachromosomal circular protein, laterally conjugally transfer to non-89K *S. suis* recipients through the 89K-encoded GI-type T4SS [23]. Based on genome comparisons and evolution relationship analysis in the current...
study, we identified that this GI-type T4SS-like system is a new subgroup T4SS named as Type-IVC secretion system for its clearly different genetic organization with type-IVA and type-IVB in Gram-negative bacteria. VirB1, VirB4, VirB6 and VirD4 were proven to be the minimal key component of type-IVC secretion system. We further identified that type-IVC secretion system is unexpectedly popular in the genus Streptococcus. This system is movable with the help of transposon factors (such as Tn916), which could also mediate the conjugative transfer of DNAs and enhance bacterial pathogenicity.

Materials and Methods

1. Genome Sequences of Streptococcus

The Streptococcus strains used in the current study are listed in Supplemental Tables. Whole genome sequences of 50 strains of Streptococcus (Table 1) were downloaded from the NCBI ftp (ftp://ftp.ncbi.nih.gov/genomes). Draft genome sequences of 67 Streptococcus strains were also obtained from the NCBI database (Table S2).

We manually collected 839 gene sequences of the 12 components of T4SS (virB1–virB11 and virD4) from the NCBI and UniprotKB database [24,25].

2. Genome-wide Search for T4SS Genes and virB/D Cluster

To search for genes in the T4SS, a new program (TFSF) was used (http://viss.bioinfo-icdc.org/). This program combines the alignment algorithm approaches, prediction of protein functions, and domain evaluation to detect candidate T4SS genes (virB1–11 and virD4) in genomes of bacteria (the detailed method is shown in the Protocol S1) with good precision.

Based on the locations of identified T4SS genes (virB/D genes), we found virB/D clusters in genomes of Streptococcus. In this study, determination of virB/D clusters conformed to the following criteria: (1) the distance between two nearby virB/D genes is less than 5 kb, (2) the total length of the virB/D cluster is less than 50 kb, and (3) the number of virB/D genes in a virB/D cluster is ≥3.

3. Identification of Genomic Islands (GI) with virB/D Cluster

For co-lineage comparisons, Blast similarity searches were performed using local BLAST software [26]. Based on the Blast results, the GI and its precise location in the genome were determined by the co-lineage comparison between a genome with a virB/D cluster and one without it [27]. The GC content skew of GI was step-analyzed in a window of 2,000 bp by a self-developed Perl program (draw_GC_content.pl) [27]. The function of genes in the GI was annotated by the method of Clusters of Orthologous Groups (COG) [28].

4. Construction of Phylogenetic Tree

rpoB is a housekeeping gene and highly conserved in many bacteria. We used rpoB gene sequences in 50 species to generate a phylogenetic tree of Streptococcus. Multiple sequence alignments of these gene sequences were performed using MEGA [29]. A phylogenetic tree was constructed using the neighbor-joining algorithm in Mega, and 1,000 subsets were generated for bootstrapping re-sampling of the data. Another tree was built based on the concatenated sequences of virB4, virB6, and virD4 genes using the same method.

Results

A New Subgroup of T4SS in Gram-positive S. suis: Type-IVC Secretion System

A new subgroup of T4SS (GI-type T4SS) was identified in Gram-positive strain S. suis [18,23]. Genetic organization represented that this GI-type T4SS in Gram-positive strain S. suis is clearly different with type-IVA, type-IVB and other T4SS in Gram-negative bacteria, thus it is classified as Type-IVC secretion system in this study (Figure 1). Different with other T4SS systems, only 4 proteins (VirB1, VirB4, VirB6, and VirD4) were identified in Type-IVC secretion systems, which mainly work in three fields: (1) transglycosylases (VirB1), working for degrading peptidoglycan outside the plasma membrane of bacteria, could reduce the resistance for the secretion of substrates; (2) ATPases (VirB4, and VirD4) play essential roles in supplying the energy for substrates translocation and apparatus assembly [4]; (3) a gene contributing to the assembly of the secretion channel across inner cell membrane (VirB6). These genes clustered together with the same direction in the chromosome. Those genes correspondent to channel subunit across outer membrane, such as VirB7/VirB9/ VirB10 in type-IVA and DotD/DotC/DotH/DotF in type-IVB, were lost in type-IVC secretion system (Figure 1 and Figure 2).

Popular Existence of Type-IVC Secretion System in Genus Streptococcus

To more fully understand the distribution of Type-IVC secretion system in genus Streptococcus, we used bioinformatic methods to predict T4SS genes (Figure S2) in 50 Streptococcus strains with published genome sequences (Table 1). Detailed methods are described in the Method section. 15 Type-IVC secretion systems were identified in 14 of 50 Streptococcus strains (Table S1). S. suis BM407 (NC_012926) has two copies of T4SS, while the other 13 genomes have only one. All 15 Type-IVC secretion systems own clustered virB1-like/virB4/virB6/virD4 or virB4/virB6/virD4 genes in the same direction and order (Table S1 and Figure S2). The Type-IVC secretion system in S. pyogenes MGAS2096 also has homolog of virB2 gene. Our genome analysis represent that, most strains in Streptococcus were identified with virB/D genes (Figure S1) and approximately 28% of all Streptococcus strains have Type-IVC secretion system (Table S1).

Additional investigations into the 67 draft genomes of Streptococcus strains (Table S2) further supported the belief that Type-IVC secretion system are popular in Streptococcus, in these studies, 19 Type-IVC secretion systems were identified (Table S2).

Evolutionary Relationship Among Type-IVC Secretion Systems in Genus Streptococcus

Among 50 strains of Streptococcus, 14 strains own additional DNA fragments (GI, 50–89 kb) with Type-IVC secretion system by co-lineage comparisons genomic comparison. Compared with the 89 kb PAI of S. suis, the structures of these GIs are significantly variable (Figure S2); Their locations in the genomes are different and genes in these GIs are also variable. However, they retain some common features. First, all the GIs have virB/D gene clusters with similar genes. Second, genes in the virB/D clusters always have the same order and direction. Third, most of the clusters own a transposon, such as Tn916.

The phylogenetic tree obtained from the housekeeping gene rpoB clearly showed that Type-IVC secretion system could be present or absent in different strains of a species (Figure 3A). The occurrence of Type-IVC secretion system in these branches could...
Table 1. List of *Streptococcus* strains with whole genomes used in the current study.

| Species                    | Strain Number | Strain Name                                                                 |
|----------------------------|---------------|-----------------------------------------------------------------------------|
| *Streptococcus agalactiae* | 3             | *Streptococcus agalactiae* 2603V R uid57943                                |
|                            |               | *Streptococcus agalactiae* A909 uid57935                                   |
|                            |               | *Streptococcus agalactiae* NEM316 uid61585                                 |
| *Streptococcus dysgalactiae* | 1            | *Streptococcus dysgalactiae* equisimilis GGS 124 uid59103                  |
| *Streptococcus equi*       | 3             | *Streptococcus equi* 4047 uid59259                                         |
|                            |               | *Streptococcus equi* zooepidemicus MGCS10565 uid59263                     |
|                            |               | *Streptococcus equi* zooepidemicus uid59261                                |
| *Streptococcus galolyticus* | 2             | *Streptococcus galolyticus* ATCC BAA 2069 uid63617                         |
| *Streptococcus gordonii*   | 1             | *Streptococcus gordonii* Challis substr CH1 uid57667                       |
| *Streptococcus mitis*      | 1             | *Streptococcus mitis* B6 uid46097                                          |
| *Streptococcus mutans*     | 2             | *Streptococcus mutans* NN2025 uid46353                                     |
|                            |               | *Streptococcus mutans* UA159 uid57947                                     |
| *Streptococcus pneumoniae* | 14            | *Streptococcus pneumoniae* 670 6B uid52533                                 |
|                            |               | *Streptococcus pneumoniae* 70585 uid59125                                 |
|                            |               | *Streptococcus pneumoniae* AP200 uid52453                                 |
|                            |               | *Streptococcus pneumoniae* ATCC 700669 uid59287                            |
|                            |               | *Streptococcus pneumoniae* CGSP14 uid59181                                |
|                            |               | *Streptococcus pneumoniae* D39 uid58581                                   |
|                            |               | *Streptococcus pneumoniae* G54 uid59167                                   |
|                            |               | *Streptococcus pneumoniae* Hungary19A 6 uid59117                          |
|                            |               | *Streptococcus pneumoniae* JJA uid59121                                   |
|                            |               | *Streptococcus pneumoniae* P1031 uid59123                                 |
|                            |               | *Streptococcus pneumoniae* R6 uid57859                                    |
|                            |               | *Streptococcus pneumoniae* Taiwan19F 14 uid59119                           |
|                            |               | *Streptococcus pneumoniae* TCH8431 19A uid49735                           |
| *Streptococcus pyogenes*   | 13            | *Streptococcus pyogenes* M1 GAS uid57845                                  |
|                            |               | *Streptococcus pyogenes* Manfredo uid57847                                 |
|                            |               | *Streptococcus pyogenes* MGAS10270 uid58571                               |
|                            |               | *Streptococcus pyogenes* MGAS10394 uid58105                               |
|                            |               | *Streptococcus pyogenes* MGAS10750 uid58575                               |
|                            |               | *Streptococcus pyogenes* MGAS2096 uid58573                                |
|                            |               | *Streptococcus pyogenes* MGAS315 uid57911                                 |
|                            |               | *Streptococcus pyogenes* MGAS5005 uid58337                                 |
|                            |               | *Streptococcus pyogenes* MGAS6180 uid58335                                 |
|                            |               | *Streptococcus pyogenes* MGAS8232 uid57871                                 |
|                            |               | *Streptococcus pyogenes* MGAS9429 uid58369                                 |
|                            |               | *Streptococcus pyogenes* NZ131 uid59035                                   |
|                            |               | *Streptococcus pyogenes* SSI 1 uid57895                                   |
| *Streptococcus sanguinis*  | 1             | *Streptococcus sanguinis* SK36 uid58381                                   |
| *Streptococcus suis*       | 5             | *Streptococcus suis* OSZYH33 uid58663                                     |
|                            |               | *Streptococcus suis* 9BHAIH33 uid58665                                    |
|                            |               | *Streptococcus suis* BM407 uid59321                                       |
|                            |               | *Streptococcus suis* P1/7 uid32235                                        |
|                            |               | *Streptococcus suis* SCB4 uid59323                                        |
| *Streptococcus thermophilus* | 3            | *Streptococcus thermophilus* CNRZ1066 uid58221                             |
|                            |               | *Streptococcus thermophilus* LMD 9 uid58327                                |
| *Streptococcus uberis*     | 1             | *Streptococcus uberis* 0140J uid57959                                      |

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not be caused by a mutation, but could perhaps be caused by several rounds of DNA acquisition from other strains. Our previous experiment proved that a GI with Type-IVC secretion system can spontaneously excise to form an extrachromosomal circular product and laterally transfer to another strain [23]. Considering the proven mobility of Type-IVC secretion system, we believe that Horizontal Transfers (HT) between species brought about the acquisition of Type-IVC secretion system in *Streptococcus*. Figure 3B further shows that *virB/D* genes in Type-IVC secretion system have higher similarity in the same species than that between two species, suggesting that the movement of GIs caused by HT occurs more easily within strains of a species than that between species.

**Discussion**

**Minimal Components of Type-IVC Secretion System**

In Gram-negative bacteria, Type-IVA secretion system usually consists of 12 components to work together just as the figure 2 shown. Thorough investigations about functions of these 12...
components showed that these genes mainly work in three fields: (1) transglycosylases (such as VirB1), working for degrading peptidoglycan outside the plasma membrane of bacteria, could reduce the resistance for the secretion of substrates; (2) ATPases (VirB4, VirD4 and VirB11) play essential roles in supplying the energy for substrates translocation and apparatus assembly [4]; (3) these are still some genes contributing to the assembly of the secretion channel across inner (VirB6 and VirB8) and outer membranes (VirB7, VirB9 and VirB10).

In Type-IVC secretion system of Streptococcus, although only four components were identified (VirB1, VirB4, VirB6, and VirD4), they still work in same three fields (Figure 2). VirB1-like protein, with CHAP domain, could encode amidohydrolases and is responsible for punching holes through the peptidoglycan outside of the gram-positive cell membrane. VirB4 and VirD4 could supply energizes necessary for the substrate transport [4]. VirB6, the inner-membrane protein, is to compose the transport channel across the cell membrane. Since the lack of the outer membrane in

Figure 3. The phylogenetic tree. (A) The neighbor-joining (NJ) tree obtained on the basis of the housekeeping gene rpoB. The red solid circles represent GIs with virB/D clusters. (B) NJ tree obtained on the basis of a concatenated sequence of virB4, virB6, and virD4 genes in GI. doi:10.1371/journal.pone.0046390.g003
Novel Type-IVC Secretion System in Streptococcus

The Type-IVC secretion system could enhance bacterial pathogenicity and mediate the injection of virulent proteins into host cells. Our previous study showed that the two component system SalK/R within the 89-kb island controls the virulence of the highly pathogenic strain S. suis 2 [23]. Recently, using NimbleGen tiling arrays, Zhu and colleagues found this 89-kb fragment in 9 other virulent S. suis 2 lineages, all of which were sampled from two recent large-scale outbreaks of human infection in China [31]. The 89-kb GI which include Tn916 with tetracycline-resistance genes of the S. suis 2 strain was proved could laterally transfer to other S. suis 2 strains with the help of Type-IVC secretion system [23]. Knockout of the 2 key components (VirD4 and VirB4) of the S. suis 2 T4SS system eliminated the lethality of the highly virulent strain and impaired its ability to trigger host immune response [30].

Conclusions

In this paper, we present evidence that the GI-type T4SS-like system we experimentally defined earlier in S. suis is unexpectedly popular in the genus Streptococcus based on an analysis of deposited genome sequences. It always located in a GI with abnormal GC content. VirB1, VirB4, VirB6 and VirD4 are the minimal key component of this system in Streptococcus. We propose that this system in Gram-positive bacteria is a new subclass of T4SS (Type-IVC secretion system). Further, it is movable with the help of transposon factors, which could mediate the conjugative transfer of plasmid DNA/transposons and enhance bacterial pathogenicity.

Supporting Information

Figure S1 Strain numbers in which virB1–virB11 and virD4 genes were identified. The blue columns are the number of Streptococcus strains with virB/D genes, whereas the red columns are the number of Streptococcus strains with virB/D clusters.

Figure S2 Genomes islands with T4SS in 10 strains of Streptococcus compared to the 89-kb GI in S. suis 05ZYH33. GC%, locations of the virB/D genes, Tn916, and other important genes in GI are shown. A) S. suis SC84; B) S. suis BM407 (1000794-1091159); C) S. suis BM407 (499472-585444); D) S. pneumoniae P1031; E) S. pneumoniae G54; F) S. pneumoniae ATCC 700669; G) S. pneumoniae CGSP14; H) S. agalactiae 2603V; I) S. agalactiae NEM316; J) S. pyogenes MGA2096. Genes with varying functions are presented in different colors.

Table S1 List of virB/D clusters identified in 14 Streptococcus strains. ID: virB/D cluster ID in a genome; S: start site of virB/D gene in genome; E: end site of virB/D gene in genome; D: direction of virB/D gene.

Table S2 List of Streptococcus strains with draft genomes used in this study. “+” indicates that there is an identified virB/D gene cluster in this strain.

Protocol S1 Detailed Material and Methods.
Author Contributions
Conceived and designed the experiments: CC GFG. Analyzed the data: WZ. Contributed reagents/materials/analysis tools: WZ CR. Wrote the paper: WZ. Prepared the figures: CR. Edited the manuscript: CR.

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