The type VI secretion system protein AsaA in *Acinetobacter baumannii* is a periplasmic protein physically interacting with TssM and required for T6SS assembly

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Type VI secretion system (T6SS) is described as a macromolecular secretion machine that is utilized for bacterial competition. The gene clusters encoding T6SS are composed of core *tss* genes and *tag* genes. However, the clusters differ greatly in different pathogens due to the great changes accumulated during the long-term evolution. In this work, we identified a novel hypothetical periplasmic protein designated as AsaA which is encoded by the first gene of the T6SS cluster in the genus *Acinetobacter*. By constructing *asaA* mutant, we delineated its relative contributions to bacterial competition and secretion of T6SS effector Hcp. Subsequently, we studied the localization of AsaA and potential proteins that may have interactions with AsaA. Our results showed that AsaA in *Acinetobacter baumannii* (*A. baumannii*) localized in the bacterial periplasmic space. Results based on bacterial two-hybrid system and protein pull-down assays indicated that it was most likely to affect the assembly or stability of T6SS by interacting with the T6SS core protein TssM. Collectively, our findings of AsaA is most likely a key step in understanding of the T6SS functions in *A. baumannii*.

*Acinetobacter baumannii* (*A. baumannii*) is an important Gram-negative opportunistic pathogen which is commonly found in soil, water and on human skin. As an important species of *Acinetobacter*, *A. baumannii* is one of the most commonly isolated Gram-negative bacteria in clinical isolates. *A. baumannii* infections are becoming more and more difficult to treat due to multi- and pan-drug resistant strains\(^1\).

The type VI secretion system (T6SS) is a recently described specialized secretion machinery used by a wide variety of Gram-negative bacteria to target against both prokaryotic and eukaryotic competitors\(^2\)–\(^5\). The system consists of several proteins forming a needle like structure\(^6\) that grants Gram-negative bacteria the capacity to translocate substrates such as phospholipases, peptidoglycan hydrolases, nucleases, and membrane pore-forming proteins to neighboring cells in order to kill\(^7\). It has been well demonstrated that T6SS can mediate interbacterial competitions and thus give the bacteria growth advantages to settle in natural habitats\(^8\). It is worth mentioning that more recently, some studies revealed that the competitions between bacteria mediated by T6SS can also foster horizontal gene transfer (HGT)\(^9\)–\(^10\). A study from Cooper et al. indicated that contact-dependent neighbor killing by T6SS may be a widespread contributor to HGT, and for *Acinetobacter* in particular, killing-enhanced HGT may play a key role in the emergence of clinically pervasive MDR 'super-bug' strains\(^11\). Since there is a high incidence of antibiotic resistance in *A. baumannii*, a comprehensive study on the T6SS of *A. baumannii* may give us important clues on the pathogenesis and acquisition of antibiotic resistance of this bacteria.

The clusters encoding T6SS is composed of 13 core *tss* genes (type six subunit genes, *tssA*～*tssM*) and a variable number of *tag* genes (T6SS-associated genes) at least\(^12\). The components of T6SS are assembled in an orderly

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AsaA is required for bacterial competition. AsaA was knocked out from the A. baumannii ATCC 17978 chromosome and the mutant was named ΔasaA. Complementation using pTrc99A which is a useful vector for the expression of unfused and fused proteins in E. coli was performed to generate CΔasaA. To investigate whether asaA knock-out would affect the growth of A. baumannii, the ΔasaA, CΔasaA and wild type (WT) 17978 were tested for the growth rates in LB. As shown in Supplementary Fig. 1, there were no significant differences between different strains. So knocking out asaA did not affect the growth of A. baumannii in LB.

A recent study found that A. baumannii ATCC 17978 can utilize its T6SS to compete with E. coli13. To determine whether AsaA is required for the competition, we used E. coli strain JM109/pK18mob, which contained a kanamycin resistant plasmid pK18mob, as a target for bacterial competition assays. TsSM is a core component of T6SS. Previous study has shown deletion of tsSM in A. baumannii can completely abolish its ability to outcompete E. coli14. So we used ΔtsSM mutant as a negative control. WT 17978, ΔasaA, CΔasaA or ΔtsSM were incubated with E. coli and surviving E. coli were calculated. The results revealed that there were significant differences among different groups.

Results

AsaA is Acinetobacter specific and highly conserved in the species A. baumannii. Previous studies have described the gene cluster of T6SS in A. baumannii12,13. The cluster is about 20 kb, consisting of more than 20 T6SS genes. asaA is the first gene of the T6SS cluster. In this work, we used strain ATCC 17978 as a representative of A. baumannii. In a BLAST comparison of the amino acid sequences of AsaA homologues, AsaA only present in the genus Acinetobacter and the homology of the AsaA amino acid sequence differs between different species. The AsaA of A. baumannii shares 87.8–92.6% similarity with that in the species A. pittii, A. calcoaceticus and A. nosocomialis, with a much more lower similarity 46.8% and 57.8% with A. baylyi and A. indicus. In the species A. baumannii, it is highly conserved among different strains with different sequence types. The amino acid sequence of AsaA in ATCC 17978 shares 99.1–100% similarity with the other A. baumannii strains (Table S3).

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were not influenced by *E. coli* and there were no significant differences in the survival of *A. baumannii* among different groups (Fig. 1C). These results suggest that AsaA is critical for bacterial competition in *A. baumannii* ATCC 17978.

**AsaA is required for Hcp secretion.** As Hcp secretion reflects the functionality of the T6SS, detection of secreted Hcp in the culture supernatants is a marker of a functional T6SS. In order to explore the mechanisms of AsaA involved in bacterial competition, we examined whether AsaA is involved in the secretion of Hcp. RT-PCR analysis showed that *hcp* were being actively transcribed in the ΔasaA mutant cultured in LB medium (Fig. 2A). To further investigate whether AsaA is involved in Hcp secretion, we performed Western blot assays to test the secretion of Hcp in the ΔasaA background. For this purpose, the recombinant plasmid pTHcpH6 containing the *hcp* gene and the 6×His-tag coding sequence, was introduced into different strains, resulting in the strains named 17978/pTHcpH6, ΔasaA/pTHcpH6 and ΔtssM/pTHcpH6, respectively (Table S1). Hcp were present in the whole cell lysates in all the three strains (Fig. 2B), at the same time, it is present in the supernatant of 17978/pTHcpH6 but absent in ΔasaA/pTHcpH6 or ΔtssM/pTHcpH6, suggesting that deletion of asaA affected the secretion of Hcp (Fig. 2B). These results indicated that AsaA is critical for Hcp secretion.

**AsaA located in the periplasm of *A. baumannii* cells.** The protein encoded by asaA in *A. baumannii* ATCC 17978 was annotated as conserved hypothetical protein. It has 230 amino acids (aa). We performed bioinformatics analysis on AsaA and failed to find additional information about its biochemical function and any known domain or motif. According to the amino acid sequence analysis of the SignalP program (http://www.cbs.dtu.dk/services/SignalP/) and the LipoP program (http://www.cbs.dtu.dk/services/LipoP/), AsaA has a signal fragment and it may be a secreted protein, but not a lipoprotein. This suggested that AsaA might be located in the periplasm or secreted outside the cells. To determine the cellular location of AsaA, we constructed a ΔasaA/pTasaAH6 strain, which expressed AsaA with a 6×His-tag. The total cell lysates, inter membrane, periplasmic, outer membrane and extracellular protein fractions of ΔasaA/pTasaAH6 cultured at logarithmic growth phase were prepared. As shown in Fig. 3, using the inner membrane protein PglC, the periplasm protein DsbA, the outer membrane protein OmpA, the extracellular protein Hcp, and the cytoplasm protein Dnak as controls, we found that AsaA is present in the periplasmic and total cell lysates but absent in the extracellular, inter membrane or outer membrane protein fractions, indicating that AsaA located in the periplasm of *A. baumannii*.

**AsaA interacts with TssM.** Based on the facts that AsaA located in the bacterial periplasm and takes part in Hcp secretion but not involved in Hcp expression, we presumed that AsaA might physically interacts with the T6SS components. To this end, we performed bacterial two-hybrid system to investigate the possibility of the physical interactions between AsaA and a series of the T6SS core proteins: TssB, TssL and TssM. In ATCC 17978, the gene encoding TssM was divided into two open reading frames, *A1S_1302* and *A1S_1303*. *A1S_1302* encodes the N-terminus and the middle part of TssM (we named this part TssM1302), *A1S_1303* encodes the C-terminus of TssM (we named this part TssM1303). In this study, we tested the interactions between AsaA with TssM1302(33–415) (the 33 to 415 amino acids of TssM1302) with TssM1302(436–1041) (the 436 to 1041 amino acids of TssM1302).
to 1041 amino acids of TssM1302 which composite the periplasmic domain of TssM and TssM1303. The open reading frame of asaA excluding the N-terminal leader was fused to pBT, yielding a plasmid named as pBA. The open reading frames encoding TssB, TssL, TssM1302(33-415), and TssM1302(436-1041), were fused to pTRG, yielding recombinant plasmids named as pTB, pTL, pTM1302(33-415) and pTM1302(436-1041) respectively. Finally, the plasmids were co-introduced into XL1-Blue MRF′ strain. For confirmation, all the resulting recombinant strains were tested on dual-selective medium containing 3-AT and streptomycin. If interactions between the proteins occur, the recombinant strains can obtain the ability to grow on dual-selective medium due to the activation of HIS3-addA reporter genes. Our results showed that only the X/pBA-pTM1302(436-1041) can grow well on dual-selective medium, in contrast, the other recombinant strains can’t grow on dual-selective medium (Fig. 4A), implying that physical interactions existed between AsaA and the periplasmic domain of TssM in the reporter strain.

To validate the interaction between AsaA and TssM1302(436–1041), the pull-down biotinylated protein-protein assay was further performed. The AsaA25–230 (from the 25th to 230th aa) and TssM1302(436–1041) encoding sequences were cloned into the expression vector pET-30a to produce the recombinant His-tagged proteins AsaA-His6 and TssM-His6 (Fig. 4B, full-length gel was presented in Supplementary Fig. 2). AsaA-His6 was immobilized on sepharose beads, and then pull-down assays for TssM-His6 was performed. As shown in Fig. 4C (full-length gel was presented in Supplementary Fig. 3), the AsaA-His6 protein was able to capture TssM-His6 protein (lane 1). Vice versa, TssM-His6 protein immobilized on streptavidin sepharose beads was also able to capture AsaA-His6 protein (lane 3). So, AsaA-His6 and TssM-His6 can capture each other. The results indicated that AsaA interacts directly with TssM1302(436–1041).

We then tried to find the exact domains of AsaA that interacts with TssM1302(436–1041). Since the first 24 amino acids of AsaA encode a signal peptide, we used the truncated AsaA excluding the N-terminal signal peptide sequence for testing. DNA fragments as shown in Fig. 5A encoding the different domains of AsaA (the 25th–70th aa, 25th–110th aa, 25th–150th aa, 25th–190th aa, 70th–230th aa, 110th–230th aa, 150th–230th aa and 190th–230th aa) were amplified and fused to pBT, respectively. Then, the recombinant plasmids and pTM1302(436-1041) were co-introduced into XL1-Blue MRF′ strain, respectively. All the resulting recombinant strains were tested on dual-selective medium. Only the strains which harbored the plasmid pBA25–150-pTM1302(436-1041), pBA70–150-pTM1302(436-1041) and pBA70–230-pTM1302(436-1041) could grow on the dual-selective medium but others could not (Fig. 5B). This indicated that the 70th–150th aa of AsaA were crucial for the interaction. We further tested the interactions of the 70th–150th aa, 70th–110th aa, and 110th–150th aa of AsaA with TssM1302(436-1041). Only the strain containing pBA70–150-pTM1302(436-1041) could grow on the selective agar plates as shown in Fig. 5B. The pull-down biotinylated protein-protein assay was performed for further validation. As shown in Fig. 5C, TssM-His6 protein was able to capture AsaA70–150-His6 protein (lane 1). Vice versa, AsaA70–150-His6 was also able to capture TssM-His6 protein (lane 3). Our results indicated that the peptide of AsaA consisting of aa from the 70th to the 150th is sufficient to interact with the periplasmic domain of TssM.

**Figure 3.** Subcellular localization of AsaA as determined. Cellular fractionation studies of AsaA in cytoplasmic (Cyto), periplasmic (Peri), inner membrane (IM), outer membrane (OM) and extracellular (Extr) fractions of *A. baumannii* with PglC, DsbA, OmpA, Hcp and DnaK as controls.
Discussion
Our knowledge about the molecular architecture and the function of T6SS has great strides. For *A. baumannii*, several studies have been performed to elucidate its functions. It has been proven that the T6SS of *A. baumannii* is responsible for bacterial competition and is also implicated in host colonization. However, for the
functions of the proteins in T6SS, only a few were approved experimentally, including VgrG which contributes to both virulence and antimicrobial resistance in A. baumannii

In this study, we made a detailed study on the function of AsaA. AsaA is encoded by the first gene within the T6SS cluster. By comparative bioinformatics analysis, we found AsaA is specific to the genus Acinetobacter and is highly conserved in the species A. baumannii. Studies on the function of the highly conserved and Acinetobacter specific T6SS protein AsaA can help us learn more about the pathogenesis of A. baumannii. We used ATCC 17978 as a representative of A. baumannii to study whether AsaA is critical for bacterial competition. The competition ability of ATCC 17978 varied with different prey cells.

**Figure 5.** Determination of the AsaA fragment required for the interaction with TssM. (A) Different AsaA fragments tested for the interaction with TssM. YES, fragment can interact with TssM; NO, fragment can't interact with TssM. (B) Bacterial two-hybrid assays. The X/pBA-pTM strain was used as positive controls, the X/pB-pTM strain was used as negative control. (C) Pull-down assays. Lanes: 1, pull-down of His₆-AsaA by immobilized His₆-TssM; 2, His₆-AsaA by immobilized streptavidin sepharose beads (negative control); 3, pull-down of His₆-TssM by immobilized His₆-AsaA by immobilized streptavidin sepharose beads (negative control); M, molecular mass marker.
E. coli DH5α, while Weber et al. showed that ATCC 17978 is unable to utilize its T6SS for antibacterial activity against E. coli MG1655. We postulated that different E. coli strains may have different immunity proteins and thus resulted in different competition abilities. In this study, we found ATCC 17978 can well compete E. coli JM109 and AsaA is critical for the competition.

Our results demonstrated that AsaA localizes in the periplasm, where it interacts with the periplasmic domain of TssM by a 80 aa peptide from the 70th to 150th aa, forming a two protein complex. AsaA is not participated in the regulation of the Hcp expression but is essential for Hcp secretion in A. baumannii ATCC 17978. At this stage, AsaA played an important role in the T6SS, but its precise roles remain to be determined. However, based on the facts that (1) mutants of AsaA and TssM showed similar phenotypes in bacterial competition and Hcp secretion; (2) similar to tssM, asaA is within the T6SS gene cluster; and (3) the TssM (A1S_1302/436–1044) extends into the periplasm and interact with AsaA physically, we presume that AsaA is most likely to be a structural component of the T6SS. The interaction with TssM may consolidate the structure of T6SS and thus facilitate the transportation of effectors.

The T6SS structural components are encoded by T6SS cluster, which are probably acquired by horizontal gene transfer during evolution. It has been shown that A. baumannii T6SS clusters appear to be conserved amongst sequenced A. baumannii strains with the exception that a portion of the gene cluster is inverted. However, the clusters have undergone great changes during the long-term evolution. A main difference from other bacteria is that the genus Acinetobacter do not produce a readily identifiable homolog of core-components TssJ, which has been shown to interact with the extreme C-terminus of TssM. Whether AsaA can replace TssJ is of interest. In this study, we found that: (1) AsaA does not interact with TssM (A1S_1302), which encode the C-terminus of TssM, but interact with TssM (A1S_1302), which encode the N-terminus and the middle part of TssM; (2) AsaA localizes in periplasm, while TssJ localizes in outer membrane; and (3) secondary structure of AsaA is predicted to be alpha helix, while TssJ is mostly beta-folded. Base on these facts, we presume that AsaA is unlikely a replacement protein for TssJ.

Contact-dependent growth inhibition (CDI) system are alternative war machines doing the same job as T6SS, i.e. fight against neighbouring cells and oucompeting them. They are found on the cell surface of both A. baumannii and A. baylyi ADP1 cells. Only a relatively few STs host CDI system in A. baumannii, the same holds for T6SS cluster and asaA gene, whether there is a correlation between the presence/absence of T6SS and CDI system need further investigation.

In conclusion, we confirmed that AsaA is required for the secretion of Hcp and it most likely affects the assembly or stability of the T6SS by interacting with the periplasmic domain of the core T6SS protein TssM in the periplasmic space. We cannot exclude the possibility that AsaA associates with assisting the apparatus assembling or affecting the apparatus stability rather than as a T6SS structural component. Nonetheless, given the fact that AsaA is an Acinetobacter genus-specific protein, the results suggest that the T6SS structural components of Acinetobacter is distinctive from other Gram-negative pathogens.

Methods

Strains and culture conditions. All strains and plasmids are summarized in Table S1. The reporter strains were grown in M9 His-dropout medium, other strains were grown in LB medium. Antibiotics, when appropriate, were added to bacterial cultures medium at the following final concentrations in µg/mL: chloramphenicol (Cm) at 12.5, streptomycin (Sm) at 10, ampicillin (Amp) at 100, tetracycline (Tc) at 20, and kanamycin (Kan) at 50.

Construction of mutant and complement strains. All primers used in this study are summarized in Tables S2. asaA and tssM gene knock out mutants of A. baumannii ATCC 17978 were constructed with the method described by Tracy and associates. Briefly, a kanamycin resistance cassette was amplified with a pair of 76bp oligonucleotide primers with 56 nucleotides of homology to the flanking regions of the targeted gene and an additional 20 nucleotides of homology to kanamycin resistance cassette from plasmid pKD46. Subsequently, the PCR products were electroporated into A. baumannii ATCC 17978 carrying pKD46 plasmid, then clones were screened on kanamycin containing LB agar plates. Genomic DNA was isolated from kanamycin resistant clones, amplicons were prepared using primer set 2 and confirmed by sequencing. To remove the kanamycin resistance cassette, pCP20 expressing recombinase was transformed into the mutants. The asaA and tssM gene knock out mutants were confirmed by polymerase chain reaction (PCR) and sequencing.

For genetic complementation of mutant strains, the ORF (open reading frame) sequence of asaA (gene number: A1S_1292) and tssM (gene number: A1S_1302) were amplified from A. baumannii ATCC 17978. Subsequently, the PCR products were cloned into pTrc99A with confirmation by sequencing.

Bacterial growth assays. Bacterial growth assays was performed as previously described by Salomon and associates. Triplicates of A. baumannii strains grown overnight in LB were normalized to OD600 of 0.01, and the growth was monitored in LB of cultures incubated at 37°C. Experiments were repeated at least twice with similar results. A representative experiment was shown.

Bacterial competition assays. Bacterial competition was performed as previously described by Brent and associates. The pl18mob plasmid was transformed into E. coli strain JM109, generating the strain named JM109/pK18mob. Cultures of A. baumannii and JM109/pK18mob were grown overnight, and the JM109/pK18mob was washed three times with PBS to remove kanamycin. Cultures were diluted to OD600 of 1.0. Then 100 µl E. coli was mixed with 10 µl A. baumannii, and 10 µl of the mixture was spotted onto a LB agar plate. After 12 h incubation at 37°C, spots were excised from the LB agar, the bacteria were diluted serially with 10-fold. Dilutions were plated onto kanamycin containing LB agar plates to select for E. coli and onto non-antibiotic
proteins. The cell was disrupted by sonication and further centrifuged at 11 000 g was then centrifuged at 130 000 g sodium lauryl sarcosine and centrifuged at 130 000 g membrane proteins. All samples were analyzed by Western blot analysis by probing with antibodies to the 6tag (Hcp, AsaA, OmpA, DsbA and PglC) and DnaK34.

The final protein was eluted by elution buffer. Finally, the final protein was separated by electrophoresis followed by coomassie blue staining.

The representative experiment was shown.

**Hcp secretion assays.** pTrc99A-hcp recombinant vector expressing His-tagged Hcp was transformed into A. baumannii ATCC 17978. Cultures were grown in LB with ampicillin to exponential phase. Expression of Hcp was induced by 1 mM IPTG for 2 h. Then 10 ml cultures were centrifuged at 10 000 × g for 10 min to obtain whole cell pellets. The supernatant containing secreted Hcp protein were filtered through a 0.22 μm pore filter. A mixture of 8.5 ml of supernatant and 1.5 ml of 100% trichloroacetic acid (TCA) was placed on ice for 4 h and centrifuged at 10 000 × g for 20 minutes at 4 °C. Precipitated proteins were washed with 100% acetone and then re-suspended in 5 ml PBS. Subsequently, protein concentrations were measured using a BCA protein assay kit. Equal amounts of total protein (25 μg) were used for polyacrylamide gel electrophoresis and proteins were analyzed by Western blot31. Experiments were repeated at least twice with similar results. A representative experiment was shown.

**Cellular localization of AsaA.** The cell fractionations were prepared as described by Zang et al.32 and Deng et al.33, with minor modifications. Briefly, overnight cultures of the cells were re-cultured with 1:10 and grown until an OD600 of 0.5 was reached. Then, the cells were collected by centrifugation at 4 000 × g for 10 min and the supernatant was filtered through a 0.22 μm pore filter and reserved as the extracellular proteins. The cell pellets were washed three times and re-suspended in 1 ml of periplastic buffer (30 000 U of lysozyme, 20% sucrose, 1 mM EDTA) and incubated on ice for 10 min. After centrifugation, the pellet was re-suspended in 10 mM magnesium chloride and incubated at 30 °C for 5 min, and then incubated at 0 °C for 10 min. The sample was freeze-thawed and pelleted by centrifugation, and then the supernatant was reserved as the periplasmic proteins. The cell was disrupted by sonication and further centrifuged at 11 000 × g for 30 min. The supernatant was then centrifuged at 130 000 × g for 1.5 h to isolate total membranes. The pellet was suspended in 0.25% (w/v) sodium lauryl sarcosine and centrifuged at 130 000 × g for 1.5 h. The supernatant was reserved as the inner membrane proteins. The pellet was re-suspended in TM buffer (8 mM MgSO4, 10 mM Tris) and reserved as the outer membrane proteins. All samples were analyzed by Western blot analysis by probing with antibodies to the 6His tag (Hcp, AsaA, OmpA, DsbA and PglC) and DnaK34.

**Western blot.** Proteins were resolved onto PVDF membranes. Membranes were blocked in 5% non-fat milk in 1× TBST buffer for 1 h at room temperature. Membranes were incubated overnight at 4 °C with anti-His, or anti-Dnak at a 1:1000 dilution. After washed with 1× TBST buffer for five times, the membrane was incubated with a HRP-conjugated secondary antibody for 50 min at room temperature. Membranes were then washed five times in 1× TBST buffer. Hybridizing bands were detected using the ECL kit.

**Bacterial two-hybrid assays.** Bacterial two-hybrid assays in vivo were tested using the BacterioMatch® II two-hybrid system (Stratagene). The truncated asaA, tssM and other genes were amplified by PCR with corresponding primers in Table S2. The amplified asaA gene excluding the N-terminal leader was fused to the vector pBT, yielding the plasmid pBA. The amplified tssM and other genes and their truncated derivatives were individually fused to the vector pTRG, respectively, yielding the plasmids pTM1302 (33–415)–pTM1302 (436–1041)–pTRG, pTM1303 and pTL (Table S1).

In order to explore the interaction between AsaA and TssM, 120, 240, 360, 480 bp and other fragments containing partial asaA were fused to pBT, respectively. Pairs of plasmids were co-transformed into XL1-Blue MRF', and the transformants were cultured on M9 His-dropout medium plate containing Sm (12.5 μg/ml) and 3-AT (5 mM) at 30 °C for 24 h. Overproduce and purification of recombinant protein. To overproduce the truncated peptides of AsaA and TssM, fragments coding sequence were fused to PET-30a. Recombinant plasmids were transformed into BL21 (DE3) cell (Table S1). The recombinant strains were grown to an OD600 of 0.4, and then 1 mM IPTG was added to induce the expression of individual proteins. Cells were harvested and washed with PBS buffer, and broken by sonication. The recombinant proteins were purified by Ni-NTA His-bind® (Novagen), as described in the manual.

**Pull-Down assays.** To analyze the interaction between AsaA with TssM, pull-down assays were tested using the ProFoundTM pull-down biotinylated protein-protein interaction kit (Pierce). As described by li and associates35, the AsaA protein was biotinylated by sulfo-NHS-LC-biotin. Subsequently, the biotinylated AsaA was incubated with streptavidin sepharose™ beads. The beads were washed four times and sample containing 60 μg of TssM protein was added and incubated. The beads were washed four times with washing buffer. Subsequently, the final protein was eluted by elution buffer. Finally, the final protein was separated by electrophoresis followed by coomassie blue staining.
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Author Contributions

F.M.L. and Q.H.Z. conceived and designed the study. L.L., Y.N.W., H.B.J., P.W., J.F.D. and J.D. performed experiments and analyzed the data. L.L. and Q.Z. wrote the manuscript. All the authors contributed to discussion and manuscript preparation.

Additional Information

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