Recent Advances in Streptococcus suis Type 2 Virulence Factors Research: Postprint

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Abstract

Streptococcus suis is an infectious Gram-positive bacterium and an important zoonotic pathogen that seriously impacts the development of the swine industry, causing human mortality rates ranging from 5% to 20%. Its virulence factors play an important role in the pathogenic process. In recent years, research on virulence factors of Streptococcus suis serotype 2 has made significant progress, yielding novel insights into its pathogenic mechanisms and effective prevention and control strategies. This article summarizes and analyzes recent progress in studies on virulence-related factors of Streptococcus suis serotype 2, covering new developments in protein and enzyme research, as well as advances in two-component systems regulating virulence factor gene expression and type IV secretion systems that interact with the host immune system, aiming to provide new references for the treatment of Streptococcus suis disease and vaccine development.

Full Text

New Progress in the Study of Streptococcus suis Type 2 Virulence Factors

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Abstract

Streptococcus suis is an infectious Gram-positive bacterium and an important zoonotic pathogen that severely impacts the swine breeding industry, causing human mortality rates between 5% and 20%. Its virulence factors play crucial roles in pathogenesis. Recent years have witnessed significant new advances in research on the virulence factors of Streptococcus suis type 2 (SS2), providing

novel insights into its pathogenic mechanisms and effective prevention and control strategies. This review summarizes recent progress in SS2 virulence-related factors, focusing on new developments in protein and enzyme studies, as well as advances in two-component systems regulating virulence gene expression and Type IV secretion systems that interact with the host immune system, aiming to provide new references for the treatment of streptococcal disease and vaccine development.

Keywords: Streptococcus suis type 2; Virulence factors; Pathogenesis

Streptococcus suis type 2 (SS2) is a facultatively anaerobic, Gram-positive, hemolytic coccus and a zoonotic pathogen. In pigs, infection can cause meningitis, arthritis, abortion, abscesses, septicemia, infectious endocarditis, and death [1-3]. Human infection with SS2 can lead to meningitis, endocarditis, and toxic shock syndrome. In 1998, an epidemic of swine streptococcal disease broke out in parts of Jiangsu Province, causing large numbers of pig deaths and infecting 25 people, of whom 14 died—13 from toxic shock syndrome. In 2005, a large-scale public health event involving SS2 infection occurred in Ziyang, Sichuan, resulting in 38 human deaths. These outbreaks demonstrate that SS2 not only causes significant losses to the swine industry but also poses serious threats to the lives and property of relevant practitioners [4], attracting widespread attention both domestically and internationally [5]. Based on capsular polysaccharide antigen differences, SS is classified into 35 serotypes (1-34 and 1/2) [6], among which serotype 2 is the most prevalent and most pathogenic. Previous systematic reviews have summarized SS2 virulence factors from perspectives including polysaccharides, proteins, enzymes, and pathogenicity islands [7]. However, with increasingly detailed and in-depth research in recent years, many new advances and understandings have emerged. This review summarizes the progress over the past decade in SS2 virulence factor research, focusing on new developments in protein and enzyme studies, as well as novel findings regarding two-component systems regulating virulence gene expression and Type IV secretion systems that interact with the host immune system.

1. Protein Virulence Factors

In addition to the six well-established virulence protein factors—capsular polysaccharide, extracellular protein factor, fibronectin-binding protein, muramidase-released protein, 38 kDa bacterial surface protein, and hemolysin [7]—new virulence-associated proteins such as epidermal growth factor receptor (EGFR) and manganese protein (MntE) have been discovered.

1.1 Epidermal Growth Factor Receptor (EGFR)

EGFR initiates intracellular signal transduction and regulates host inflammatory responses. Yang et al. [8] found that when the SS2 strain SC19 infects human brain microvascular endothelial cells (hBMEC), it induces tyrosine phosphorylation of cellular EGFR in a ligand-dependent manner involving EGF-

like ligands HB-EGF, amphiregulin (AREG), and epiregulin (EREG), leading to EGFR/ErbB3 heterodimerization. EGFR transactivation does not participate in SS2 strain SC19 adhesion to hBMEC or bacterial colonization in vivo. However, this transactivation promotes bacteria-induced neuroinflammation by triggering MAPK-ERK1/2 and NF- B signaling pathways in hBMEC, thereby enhancing production of pro-inflammatory cytokines and chemokines. These observations reveal a novel mechanism involving EGFR in SS2-mediated inflammatory responses in the brain, suggesting that EGFR may be an important host target for further research and prevention of neuroinflammation caused by SS2 strains.

1.2 Manganese Protein (MntE)

Manganese is an essential micronutrient for bacteria that plays important roles in physiological functions, but excess manganese is highly toxic to cells. Manganese efflux systems control intracellular manganese levels in some bacteria. Xu et al. [9] identified a cation diffusion facilitator protein (MntE) and constructed an mntE deletion mutant (ΔmntE) and complementary strain (CΔmntE). Compared with wild-type and complementary strains under normal growth conditions, ΔmntE showed similar growth but exhibited defective growth in media supplemented with high concentrations of manganese. Additionally, the mutant was more sensitive to oxidative stress conferred by diamide. Using competitive infection assays in a mouse infection model, the study demonstrated for the first time that MntE contributes to the virulence expression of SS2, indicating that manganese homeostasis controlled by the manganese efflux system MntE plays an important role in SS2 pathogenesis.

1.3 Host Specificity Determinant Specificity Subunit (hsdS) of Type I Restriction-Modification System

Resistance to phagocytosis and survival in whole blood are essential requirements for bacterial pathogenesis. SS2 regulates peptidoglycan-binding proteins by modulating the level of the host specificity determinant specificity subunit (hsdS) of the Type I restriction-modification system, thereby promoting anti-phagocytic effects against microglial cells (BV2). Statistical analysis of characteristic differences between gene mutants and wild-type SS2 revealed that under non-acidic conditions, their survival in BV2 cells, whole blood, and hydrogen peroxide environments (H O) increased significantly ($p < 0.05$). In contrast, another specificity subunit encoded by hsdS' , belonging to the same Type I restriction-modification system, only significantly reduced SS2 survival under acidic conditions in the gene deletion mutant form ($p < 0.05$) but had no significant effect or even enhanced anti-phagocytic ability under other conditions compared with wild-type SS2. Therefore, hsdS promotes bacterial anti-phagocytosis and survival in hostile host environments by positively affecting transcription of two peptidoglycan-binding protein genes, enhancing resistance to reactive oxygen species, and reducing secretion of TNF- and nitric oxide by phagocytes.

These findings reveal a novel mechanism of SS2 pathogenesis [10].

1.4 Mac Protein

Xiao et al. [11] investigated the Mac protein of SS2, which contains a Mac-1 domain, in naïve healthy hosts without specific IgM. The study constructed a mac gene deletion mutant in the SS2 virulent reference strain P1/7 through homologous recombination. Deletion of the mac gene did not substantially affect phagocytosis in mouse macrophages RAW264.7 or intracellular survival. Furthermore, the mutant showed the same virulence as the wild-type strain in pig, mouse, and zebrafish infection models. These results indicate that the Mac protein in SS2 strain P1/7 is not essential for virulence in naïve healthy hosts without specific IgM, and the immunogenicity of Mac appears to be unrelated to its virulence.

1.5 Factor H (FH)

Factor H (FH) is a regulatory protein of the complement system that specifically binds to the Factor H-binding protein (FHBP) of SS2, helping the bacteria evade host innate immune defense. Li et al. [12] selected eight newly identified FHBP proteins from SS2 and found that pre-incubation of SS2 with anti-FHBP polyclonal antibodies significantly reduced binding to immobilized FH. In a mouse intraperitoneal challenge model, SS2 not pre-treated with FH showed increased bacteremia and brain invasion compared with FH-pre-treated SS2. These results demonstrate that FH binding to the cell surface enhances SS2 adhesion to and invasion of HEp-2 cells, promoting SS2 survival in the host through anti-phagocytic effects.

1.6 Cyclic AMP Receptor Protein (cAMP)

SSU05-0736 encodes a cAMP receptor protein that functions as a transcriptional regulator belonging to the Crp/Fnr family. A gene deletion mutant (Δ0736) was constructed using homologous recombination. While no significant differences in biological activity or virulence were observed between the mutant and wild-type strains, the mutant exhibited slower growth and a larger growth rate. The cAMP receptor protein is not a direct determinant of SS2 virulence but may potentially regulate SS2 virulence by transcriptionally activating carbon metabolism-related proteins that affect bacterial metabolic functions, indicating that it is also a potential virulence-associated protein [13].

1.7 CodY and SPX Domain Proteins

CodY and SPX are both global transcriptional regulators that are highly conserved in low-GC Gram-positive bacteria. CodY is a conserved dimeric structure composed of two 29 kDa subunits, belonging to the LacI protein family, with two effectors—guanosine triphosphate (GTP) and branched-chain amino acids (BCAA)—and participates in bacterial nitrogen and carbon metabolism.

In SS2, deletion of the codY gene resulted in decreased adhesion and invasion capabilities in Hep-2 cells. In RAW246.7 cells, the codY mutant strain was rapidly cleared by macrophages, demonstrating that CodY is a virulence factor of SS2 [14]. The SPX domain is named from the initials of proteins in yeast (SYG1, Pho81) and humans (XPR1) that contain a relatively conserved common domain at the amino terminus [15-17] and represents a group of global transcriptional regulators. Zhang et al. [18] constructed gene deletion mutants SpxA1 and SpxA2 and found that both mutants exhibited phenotypic changes and significantly reduced virulence compared with the wild-type strain, indicating that SPX domain proteins also play important roles in SS2 pathogenesis.

1.8 Hemolysis-Related Protein (HHly3)

Li et al. [19] identified a novel hemolysis-related protein, HHly3, on the 89K pathogenicity island. This 78 kDa protein is a membrane channel protein belonging to the hemolysin III superfamily. Sequence analysis of its amino acid coding sequence revealed conserved sequences identical to those of Type IV secretion systems, suggesting it may participate in forming a Type IV secretion system and possess related functions such as involvement in energy metabolism, thereby potentially affecting SS2 virulence. Construction of a gene deletion mutant revealed reduced adhesion to Hep-2 cells, decreased biofilm formation capacity, and significantly attenuated virulence in zebrafish pathogenicity experiments. Western blot detection showed that recombinant HHly protein has good immunogenicity [20]. These findings demonstrate that HHly3 plays an important role in SS2 infection and represents a new virulence factor of SS2.

2. Enzyme Virulence Factors

2.1 Hyaluronic Acid Lyase (HYL)

HYL is a linear aminoglycan composed of -1-4-N-acetylglucosamine-glucuronic acid polymers that exists widely in various mammalian tissues and participates in forming the extracellular matrix. The degradation of hyaluronic acid by HYL not only provides disaccharides that bacteria can utilize for growth but also facilitates bacterial entry into host tissues by altering extracellular matrix permeability, which is clearly related to its pathogenic mechanism. Haas et al. [21] found that during infection, when SS2 interacts with host cells, HYL regulates SS2 adhesion to brain microvascular endothelial cells (BMEC), increases expression of SS2 virulence factors, and enhances pro-inflammatory factor release through BMEC.

2.2 S-Ribosylhomocysteine Lyase (Luciferase, LuxS)

LuxS is a global regulator belonging to the LuxS protein family. It is the synthase for the autoinducer-2 (AI-2) signaling molecule in quorum sensing systems and also a metabolic enzyme in the activated methyl cycle that plays important roles in metabolism. In studies of the highly virulent SS2 strain 05ZYH33, luxS

deletion caused slow growth, a thinner capsule, and other phenotypic changes, as well as decreased adhesion to Hep-2 cells. When piglets were challenged with mutant and wild-type strains, the mutant showed significantly reduced lethality and decreased colonization ability in the host, while the functional complementary strain was similar to the wild-type [22], demonstrating that LuxS is an important regulator of SS2 virulence. Wang et al. [23] also confirmed that Phe80 and His87 residues in LuxS are directly related to AI-2 activity, thereby indirectly controlling expression of some SS2 virulence genes.

2.3 N-Acetylneuraminic Acid (NeuB)

N-acetylneuraminic acid, also known as sialic acid, is primarily found in bacterial capsular polysaccharides and mediates adhesion to macrophages with anti-phagocytic functions. Dong et al. [24] constructed a neuB gene deletion mutant and complementary strain through genetic recombination. The deletion mutant exhibited a thinner capsule, pH sensitivity, decreased adhesion to laryngeal carcinoma mucosal cells (Hep-2), and significantly reduced virulence compared with the parental strain, while the complementary strain restored virulence to near parental levels. NeuB can not only induce host cells to release pro-inflammatory factors but also accelerate IL-8 secretion, and its mutant is easily cleared in whole blood [25]. Zhu et al. further confirmed that the sialic acid synthase NeuB evades phagocytosis by host macrophages by inhibiting activation of the TLR2-AKT-NF- B signaling pathway [26]. Therefore, NeuB is associated with SS2 pathogenicity and represents a new virulence factor.

2.4 Peptidoglycan Deacetylase (PgdA)

PgdA is an oxidative stress response-induced enzyme that participates in regulating N-deacetylation of peptidoglycan and belongs to glycoside hydrolase family IV. Enhanced PgdA activity causes peptidoglycan deacetylation, which renders bacteria resistant to lysozyme hydrolysis. Studies have shown that the degree of peptidoglycan acetylation determines resistance to human mucosal lysozyme [27]. The pgdA gene in SS2 consists of 1,398 base pairs, and both in vivo and in vitro experiments have shown enhanced pgdA expression during SS2 infection. Construction of a pgdA deletion mutant revealed increased sensitivity to lysozyme, enhanced phagocytosis, and significantly reduced virulence compared with the parental strain. The pgdA mutant was also rapidly cleared in infected piglets [28], demonstrating that PgdA is a virulence-associated factor of SS2.

2.5 Glutamine Synthetase (GlnA)

In microorganisms, GlnA participates in carbon and nitrogen metabolism in bacteria and eukaryotes and is a core enzyme in nitrogen metabolic pathways, involved in ammonia transformation and glutamine synthesis while providing nitrogen sources for synthesis of various amino acids. Construction of an SS2 glnA deletion mutant revealed slow growth and significantly decreased adhesion to Hep-2 cells compared with the wild-type strain. In pathogenicity studies,

the LD of the glnA mutant was 1.4×10^{1}, while that of the wild-type was 4.44×10, indicating significantly attenuated virulence. Dynamic distribution experiments of viable bacteria in tissues showed significantly reduced viable bacterial counts in various tissues and organs of mice infected with the glnA mutant. These results demonstrate that GlnA plays an important role in SS2 infection, particularly in adhesion and colonization processes [29].

2.6 Enolase (Eno)

Eno is an important enzyme in glycolysis that can directly exert toxicity on porcine brain microvascular endothelial cells (PBMEC), promote apoptosis, inhibit tight junction expression, or increase blood-brain barrier permeability by activating ERK and p38MAPK signaling pathways in PBMEC to secrete the pro-inflammatory factor IL-8 [30]. Huo et al. [31] successfully constructed high-purity hisSsEno recombinant protein and found through antibody blocking and whole blood killing models that SsEno is a potential anti-phagocytic factor of SS2 that can specifically bind human fibrinogen (hFg). Pian et al. [32] also confirmed that Eno significantly inhibits SS2 binding to human fibrinogen, enhances anti-phagocytic effects against neutrophils, and improves bacterial survival in blood. These findings indicate that Eno is a virulence-associated factor of SS2.

2.7 Superoxide Dismutase (SodA)

SodA is primarily involved in bacterial survival within phagocytes. After phagocytes engulf bacteria, they release superoxide species such as NO radicals through respiratory burst, which can damage bacterial DNA and proteins. Bacterial SodA can scavenge superoxides to protect bacteria from damage. The superoxide dismutase encodes 201 amino acids. Construction of a mutant strain revealed that the mutant was more susceptible to oxidative stress and more easily killed by the immune system than the wild-type strain in both in vitro and cellular experiments, indicating its important role in SS2 pathogenesis [33].

2.8 Endothelin-Converting Enzyme 1 (ECE1)

Endothelin-converting enzyme 1 is the rate-limiting enzyme for endothelin synthesis in vivo and regulates endothelin biological activity, belonging to the M13 family of metallopeptidases. Tan [34] identified 249 differentially expressed genes through gene expression profiling of a mutant strain, among which the downregulated gene SSU05-0153 showed the most significant change (44-fold). This gene encodes endothelin-converting enzyme 1 (ece1), consisting of 609 amino acids. Deletion of this gene did not alter SS2 reproductive capacity but reduced adhesion ability. However, animal experiments showed significantly higher survival rates in piglets infected with the mutant strain, indicating markedly attenuated virulence and proving that this gene is a new virulence-related gene of SS2.

3. Two-Component Transcriptional Regulatory Systems (TCS)

Fifteen potential two-component systems (TCS) have been identified in Chinese epidemic SS2 strains, among which five have been confirmed to be associated with virulence expression regulation: salK-salR [35, 36], ciaR-ciaH [37], ihk-ihr [38], virR/virS [39], and nisK-nisR [40]. The salK-salR knockout strain significantly downregulated expression of 26 genes and showed increased sensitivity to killing by polymorphonuclear leukocytes [35]. Surprisingly, another research team demonstrated that this TCS also regulates synthesis of the biologically active bacteriocin suicine produced by SS2 [36]. The ciaR-ciaH TCS has been shown to be required in CD-1 mouse and pig infection models [37]. Another study indicated that the Ihk/Irr TCS contributes to SS2 virulence by regulating bacterial physiological metabolism [38]. Recent research has confirmed that two TCS pairs (VirR/VirS and NisK/R) are essential for SS2 virulence.

Additionally, three orphan regulators have been identified: CovR [41], RevSC21 [36], and RevS [42, 43]. The orphan regulator RevSC21 positively regulates expression of virulence factors (e.g., mrp, sly, cps) and is required for bacterial pathogenesis [44]. In contrast, not all regulators positively correlate with bacterial virulence expression. Studies have shown that the orphan regulator CovR is negatively correlated with SS2 pathogenicity. The SS2 ΔcovR mutant exhibited a thicker capsule, stronger hemolytic activity, enhanced adhesion to epithelial cells, and increased resistance to phagocytosis by PMNs and monocytes. The mutant strain showed stronger virulence than the wild-type [41]. These phenomena indicate that two-component systems play complex roles in SS2-host interactions and pathogenesis, requiring further investigation and exploration.

4. Type IV Secretion System (T4SS)

The virD4 gene in the Type IV secretion system (T4SS) is located within the 89K pathogenicity island specific to recent epidemic SS2 strains and contributes to the development of toxic shock syndrome. Jiang et al. [45] found that deletion of virD4 resulted in reduced virulence, as evidenced by approximately 65% lower LD , lower bacterial loads in the liver and brain, and lower expression levels of inflammatory cytokines in mice or cells compared with the parental strain. The ΔvirD4 mutant was more susceptible to phagocytosis. Simulating bacterial exposure to phagocyte respiratory burst conditions, in vivo oxidative stress upregulated bacterial virD4 expression. Proteomic analysis identified ten secreted proteins with significant differences between the parental and mutant strains under oxidative stress, including peptididyl-prolyl isomerase (PrsA). SS2 PrsA expressed in Escherichia coli induced dose-dependent cell death and expression of pro-inflammatory cytokines IL-1 , IL-6, and TNF- in mouse macrophages. VirD4 may contribute to virulence through its anti-phagocytic activity, upregulated expression following oxidative stress, and involvement in increased secretion of PrsA as a cell death inducer and pro-inflammatory effector.

5. Other Virulence-Associated Factors

Glycerophosphodiester phosphodiesterase (GdpP) is a cyclic phosphodiesterase that can degrade diadenosine monophosphate. The gdpP mutant showed significant reductions in both hemolytic activity and adhesion to Hep-2 cells [46]. SS2 chorismate synthase (AroC) promotes TLR4-dependent inflammatory responses in RAW264.7 cells through the p38MAPK and NF- B pathways [47]. Glucose-inhibited division protein (GidA) is a tRNA modification enzyme. Deletion of gidA causes bacterial growth defects and a thicker capsule, reduces lethality, decreases cell adhesion, and enhances anti-phagocytic ability [48]. SSU0448 participates in N-acetylgalactosamine and galactosamine metabolism. While no significant differences in pathogenicity were observed among mutant, wild-type, and functional complementary strains, Gram staining revealed that the mutant strain showed decreased chain formation, delaying the disease process, while the complementary strain restored chain formation. This indicates that the SSU0448 gene participates in regulating SS2 chain formation, indirectly affecting SS2 pathogenicity [49].

Recent studies have identified at least nine multifunctional transcriptional regulators involved in SS virulence regulation. Five have been intensively studied: adcR [50], ccpA [51, 52], argR [53], rgg [54], and PerR [55], plus four less-characterized transcription factors: 00SSU005, treR, nadR, and scrR [56]. AdcR is a regulator of zinc transport, and its expression blockage reduces bacterial virulence in mouse infection models [50]. In contrast, another zinc uptake regulator Zur is not required for SS2 virulence in pig infection models [57]. CcpA is a transcriptional regulator that primarily mediates bacterial carbon source metabolism, which is critical for bacterial survival in the host. The ccpA mutant showed reduced growth rate, decreased colonization ability, and attenuated virulence in mouse pathogenicity tests, with a significantly thinner capsule and increased sensitivity to killing by porcine PMNs, demonstrating that the ccpA gene is associated with SS2 virulence [33, 51, 52]. ArgR is a member of the arginine repressor family and has recently been shown to regulate expression of the arcABC operon encoding arginine deiminase, thus being considered a potential virulence factor [53, 58]. The Rgg regulator, found in SS2, plays multiple roles in bacterial metabolic activities and is indispensable in porcine SS2 infection [54]. The PerR regulator has been shown to participate in SS2 virulence by regulating genes encoding hydrogen peroxide resistance protein (dpr) and methionine transporter (metQIN) [55].

These proteins and regulatory factors directly or indirectly participate in SS2 pathogenicity during infection, but how they function in the pathogenic mechanism requires further investigation.

6. Conclusion and Outlook

Streptococcus suis type 2 poses a serious threat to the swine breeding industry and human public health, and the prevention and control situation remains

severe. Many scholars have conducted extensive and in-depth research on SS2 virulence factors, with continuous reports of new potential pathogenic factors in recent years. Numerous teams have investigated the impact of virulence factors on strain pathogenicity, revealing that these related factors may not be true major virulence factors, and their presence or absence does not determine pathogenicity strength. This suggests that SS2 may possess other unknown virulence factors, and its pathogenesis may result from the combined action of multiple virulence factors with potentially complementary relationships, where partial functions of one factor can be fulfilled by another. Therefore, although academia recognizes distinctions between highly virulent, weakly virulent, and avirulent SS2 strains, no unified criteria have been established to differentiate pathogenic from non-pathogenic strains, and no 标志性 virulence factors have been identified to clearly distinguish highly virulent, weakly virulent, and avirulent strains.

Research on known SS2 virulence factors indicates that these factors may function in several aspects: anti-phagocytosis to avoid lysozyme killing [6, 12, 24-26, 28, 59]; assisting adhesion [9, 23, 29, 32, 60]; stimulating production of inflammatory cytokines [6, 59, 61]; and participating in bacterial substance and energy metabolism [1-3, 10, 11, 13, 14-17, 33]. However, many scholars now believe that these proteins may not be virulence factors per se, but their synthesis happens to be associated with virulence [62]. Moreover, do other undiscovered virulence factors exist in SS2? Therefore, future research should focus on the mechanisms of action of individual virulence factors, interactions between factors, and exploration and discovery of new virulence factors. Recent work on virulence factors has provided important references for identifying new protective antigens and investigating the biological functions of new attenuated live vaccines, laying a foundation for finding new antigens closely related to virulence, promoting deeper understanding of SS2 pathogenic mechanisms, and providing theoretical guidance and assistance for screening new therapeutic drugs and designing vaccines to facilitate scientific and rational disease prevention and control strategies.

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Note: Figure translations are in progress. See original paper for figures.