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Recent Advances in Virulence Factor Research of Streptococcus suis Serotype 2: Postprint

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Date: 2017-12-22T00:00:00+00:00

Abstract

Streptococcus suis is an infectious Gram-positive bacterium and an important zoonotic pathogen that severely affects the development of the swine industry, causing human mortality rates ranging from 5% to 20%. Its virulence factors play crucial roles in the pathogenic process. In recent years, research on virulence factors of Streptococcus suis serotype 2 has seen numerous new advances, providing new understanding of its pathogenic mechanisms and effective prevention and control of the disease. This article summarizes and analyzes recent research progress on virulence-related factors of Streptococcus suis serotype 2, including new advances in protein and enzyme studies, as well as progress on two-component systems regulating virulence factor gene expression and type IV secretion systems that interact with the host immune system, with the aim of providing 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 that represents an important zoonotic pathogen severely impacting the swine breeding industry, with human mortality rates ranging from 5% to 20%. Its virulence factors play

crucial roles in pathogenesis. Recent years have witnessed significant advances in research on Streptococcus suis serotype 2 (SS2) virulence factors, yielding new insights into pathogenic mechanisms and effective disease prevention and control strategies. This review summarizes recent progress in SS2 virulence-related factors, focusing on new developments in protein and enzyme research, as well as advances in two-component systems regulating virulence gene expression and type IV secretion systems that interact with host immune systems, aiming to provide novel references for SS2 disease treatment and vaccine development.

Keywords: Streptococcus suis serotype 2, virulence factor, pathogenesis

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—recent studies have identified novel virulence-associated proteins including epidermal growth factor receptor (EGFR) and manganese protein (MntE).

1.1 Epidermal Growth Factor Receptor (EGFR)

EGFR initiates intracellular signal transduction and regulates host inflammatory responses. Yang et al. [8] demonstrated that when SS2 strain SC19 infects human brain microvascular endothelial cells (hBMEC), it induces EGFR tyrosine phosphorylation in a ligand-dependent manner involving EGF-like ligands HB-EGF, amphiregulin (AREG), and epiregulin (EREG), leading to EGFR/ErbB3 heterodimerization. While EGFR transactivation does not participate in SS2 strain SC19 adhesion to hBMEC or bacterial colonization in vivo, it 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 findings reveal a novel mechanism involving EGFR in SS2-mediated inflammatory responses in the brain, suggesting EGFR as an important host target for further research and prevention of SS2-induced neuroinflammation.

1.2 Manganese Protein (MntE)

Manganese is an essential micronutrient for bacteria that plays important physiological roles, yet 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 mntE deletion (ΔmntE) and complementation (CΔmntE) mutants. The ΔmntE mutant exhibited similar growth to wild-type and complementation strains under normal conditions but showed defective growth in media supplemented with high manganese concentrations. Additionally, the mutant was more sensitive to oxidative stress induced by diamide. Using competitive infection assays in a

mouse model, the study first demonstrated MntE' s involvement in SS2 virulence expression, indicating that manganese homeostasis controlled by the MntE efflux system plays an important role in SS2 pathogenesis.

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

Resistance to phagocytosis and survival in whole blood are essential prerequisites for bacterial pathogenesis. SS2 regulates peptidoglycan-binding proteins by modulating levels of hsdS, the host specificity determinant specificity subunit of type I restriction-modification systems, thereby promoting anti-phagocytic activity against microglial cells (BV2). Statistical analysis of phenotypic differences between gene mutants and wild-type SS2 revealed that, under non-acidic conditions, hsdS mutants showed significantly increased survival in BV2 cells, whole blood, and hydrogen peroxide environments ($p < 0.05$). 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 activity under other conditions. Thus, hsdS promotes bacterial anti-phagocytosis and survival in adverse 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 in SS2 pathogenesis [10].

1.4 Mac Protein

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

1.5 Factor H (FH)

Factor H (FH) is a complement system regulatory protein that specifically binds to FH-binding proteins (FHBP) on SS2, facilitating evasion of host innate immune defenses. Li et al. [12] selected eight newly identified SS2 FHBPs and demonstrated that pre-incubation with anti-FHBP polyclonal antibodies significantly reduced SS2 binding to immobilized FH. In a mouse intraperitoneal challenge model, SS2 not pre-treated with FH showed increased bacteremia and brain invasion compared to FH-pre-treated SS2. These results indicate that FH

binding to the bacterial surface enhances SS2 adhesion to and invasion of HEp-2 cells, promoting SS2 survival in the host through anti-phagocytic activity.

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. Using homologous recombination to construct a gene deletion mutant (Δ0736), experiments revealed no significant differences in biological activity or virulence between the mutant and wild-type strains, though the mutant exhibited slower growth and altered growth rates. The cAMP receptor protein is not a direct determinant of SS2 virulence but may potentially regulate virulence by transcriptionally activating carbon metabolism-related proteins, indicating it represents a potential virulence-associated protein in SS2 [13].

1.7 CodY and SPX Domain Proteins

CodY and SPX are global transcriptional regulators 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)—that participate in bacterial nitrogen and carbon metabolism. In SS2, codY deletion resulted in reduced adhesion to and invasion of Hep-2 cells, and the mutant strain was rapidly cleared by macrophages in RAW246.7 cells, demonstrating that CodY is a virulence factor in SS2 [14]. The SPX domain is named from the initials of yeast (SYG1, Pho81) and human (XPR1) proteins containing a relatively conserved common domain at the amino terminus [15-17], representing a group of global transcriptional regulators. Zhang et al. [18] constructed spxA1 and spxA2 gene deletion mutants and found that both mutants exhibited phenotypic changes and significantly reduced virulence compared to 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 membrane channel protein belongs to the hemolysin III superfamily. Sequence analysis revealed conserved domains shared with type IV secretion systems, suggesting it may participate in forming a type IV secretion system with functions such as energy metabolism, thereby potentially affecting SS2 virulence. Construction of a gene deletion mutant revealed reduced adhesion to Hep-2 cells, decreased biofilm formation, and significantly attenuated virulence in zebrafish infection experiments. Western blot detection confirmed that recombinant HHly protein exhibits good immunogenicity [20]. These findings demonstrate that HHly3 plays an important role in SS2 infection and represents a novel virulence factor.

2. Enzyme Virulence Factors

2.1 Hyaluronic Acid Lyase (HYL)

HYL degrades hyaluronic acid, a linear aminoglycan polymer composed of -1-4-N-acetylglucosamine-glucuronic acid that is widely distributed in mammalian tissues and participates in extracellular matrix formation. Hyaluronic acid degradation not only provides disaccharides for bacterial growth but also facilitates bacterial entry into host tissues by altering extracellular matrix permeability, clearly implicating this process in pathogenic mechanisms. Haas et al. [21] found that during infection, when SS2 interacts with host cells, HYL regulates SS2 adhesion to brain microvascular endothelial cells (BMEC), enhances expression of SS2 virulence factors, and promotes pro-inflammatory factor release through BMEC.

2.2 S-Ribosyl Homocysteine Lyase (Luciferase, LuxS)

LuxS is a global regulator belonging to the LuxS protein family that serves as the synthase for autoinducer-2 (AI-2) signal molecules in quorum sensing systems, while also functioning as a metabolic enzyme in the activated methyl cycle, playing important roles in metabolism. Studies on the highly virulent SS2 strain 05ZYH33 revealed that luxS deletion causes slow growth, capsule thinning, and reduced adhesion to Hep-2 cells. When piglets were challenged with mutant and wild-type strains, the mutant showed significantly reduced lethality and decreased colonization capacity in the host, while the complementation strain resembled the wild-type [22], demonstrating that LuxS is an important virulence regulatory factor in SS2. Wang et al. [23] further 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 present in bacterial capsular polysaccharides, mediates adhesion to macrophages, and exhibits anti-phagocytic functions. Dong et al. [24] constructed neuB gene deletion and complementation mutants through gene recombination, finding that the deletion mutant had a thinner capsule, increased pH sensitivity, reduced adhesion to laryngeal carcinoma mucosal cells (Hep-2), and significantly attenuated virulence compared to the parental strain, while the complementation strain restored virulence to near parental levels. NeuB not only induces pro-inflammatory factor release from host cells but also accelerates IL-8 secretion, and its mutant is readily cleared in whole blood [25]. Zhu et al. further confirmed that sialic acid synthase NeuB evades macrophage phagocytosis by inhibiting activation of the host immune cell TLR2-AKT-NF- B signaling pathway [26]. Therefore, NeuB is associated with SS2 pathogenicity and represents a novel 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 protects bacteria from lysozyme hydrolysis. Research has shown that the degree of peptidoglycan acetylation determines resistance to human mucosal lysozyme [27]. The SS2 pgdA gene comprises 1,398 base pairs, and both in vivo and in vitro experiments demonstrate enhanced pgdA expression during SS2 infection. Construction of a pgdA deletion mutant revealed increased lysozyme sensitivity, enhanced phagocytosis, and significantly reduced virulence compared to the parental strain, with the pgdA strain being rapidly cleared in infected piglets [28]. These findings indicate that PgdA is a virulence-associated factor in SS2.

2.5 Glutamine Synthetase (GlnA)

In microorganisms, GlnA participates in carbon and nitrogen metabolism in bacteria and eukaryotes, serving as a core enzyme in nitrogen metabolic pathways involved in ammonia conversion and glutamine synthesis while providing nitrogen sources for various amino acid syntheses. Construction of an SS2 glnA deletion mutant revealed slow growth and significantly reduced adhesion to Hep-2 cells compared to the wild-type strain. In virulence studies, the LD50 of the glnA mutant was 1.4×10^1 , while the wild-type LD50 was 4.44×10 , indicating significantly attenuated virulence. Dynamic distribution experiments of viable bacteria in tissues showed substantially reduced bacterial loads in various 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 directly exerts toxicity on porcine brain microvascular endothelial cells (PBMEC), promotes apoptosis, inhibits tight junction expression, and increases blood-brain barrier permeability by activating ERK and p38MAPK signaling pathways to secrete the pro-inflammatory factor IL-8 [30]. Huo et al. [31] successfully constructed high-purity hisSsEno recombinant protein and, through antibody blocking and whole blood killing models, identified SsEno as a potential anti-phagocytic factor that specifically binds human fibrinogen (hFg). Pian et al. [32] also confirmed that Eno significantly inhibits SS2 binding to human fibrinogen, enhances anti-phagocytic activity against neutrophils, and improves bacterial survival in blood. These findings establish Eno as a virulence-associated factor in SS2.

2.7 Superoxide Dismutase (SodA)

SodA is primarily involved in bacterial survival within phagocytes. After phagocytosing bacteria, phagocytes undergo respiratory bursts that release superox-

ide species such as NO radicals, which can damage bacterial DNA and proteins. Bacterial SodA clears these superoxide species to prevent self-damage. The superoxide dismutase gene encodes 201 amino acids, and construction of a mutant strain revealed increased susceptibility to oxidative reactions and enhanced killing by the immune system 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, regulating endothelin biological activity and belonging to the M13 family of metallopeptidases. Tan [34] used gene expression microarray analysis of a mutant strain to identify 249 differentially expressed genes, with SSU05-0153 showing the most significant downregulation (44-fold). This gene encodes endothelin-converting enzyme 1 (ece1), comprising 609 amino acids. Deletion of this gene did not alter SS2 replication capacity or adhesion ability but significantly increased survival rates in infected piglets, indicating markedly attenuated virulence and establishing this gene as a novel virulence-associated gene in SS2.

3. Two-Component Transcriptional Regulatory Systems (TCS)

Chinese epidemic SS2 strains contain 15 putative two-component systems (TCS), of which five have been confirmed to regulate virulence expression: salK-salR [35, 36], ciaR-ciaH [37], ihk-ihr [38], virR/virS [39], and nisK-nisR [40]. The salK-salR deletion mutant significantly downregulated expression of 26 genes and showed increased sensitivity to killing by polymorphonuclear leukocytes [35]. Surprisingly, another research team demonstrated that this same TCS also regulates production of the biologically active bacteriocin suicine [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 such as mrp, sly, and cps [44] and is required for bacterial pathogenesis. Conversely, not all regulators positively correlate with bacterial virulence expression. Studies show that the orphan regulator CovR negatively correlates with SS2 pathogenicity; the ΔcovR mutant exhibits a thicker capsule, enhanced hemolysis, increased adhesion to epithelial cells, and greater resistance to phagocytosis by PMNs and monocytes, resulting in higher virulence than the wild-type strain [41]. These phenomena indicate that TCS play complex roles in SS2-host interactions and pathogenesis, requiring further

investigation and exploration.

4. Type IV Secretion System (T4SS)

The virD4 gene within the T4SS is located in 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 virD4 deletion resulted in attenuated virulence, with approximately 65% reduction in LD50, lower bacterial loads in liver and brain, and decreased expression of inflammatory cytokines in mice or cells compared to the parental strain. The ΔvirD4 mutant was more susceptible to phagocytosis. Simulating bacterial exposure to phagocyte respiratory bursts, in vivo oxidative stress upregulated virD4 expression. Proteomic analysis identified ten secreted proteins with significant differences between parental and mutant strains under oxidative stress, including peptididyl-prolyl isomerase (PrsA). SS2 PrsA expressed in Escherichia coli induced dose-dependent macrophage cell death and expression of pro-inflammatory cytokines IL-1 , IL-6, and TNF- . VirD4 may contribute to virulence through its anti-phagocytic activity, upregulated expression following oxidative stress, and involvement in secreting PrsA as a cell death inducer and pro-inflammatory effector.

5. Other Virulence-Associated Factors

Glycerophosphodiester phosphodiesterase (GdpP) protein is a cyclic phosphodiesterase that degrades 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 p38MAPK and NF- B pathways [47]. Glucose-inhibited division protein (GidA) is a tRNA modification enzyme; gidA deletion causes bacterial growth defects and capsule thickening, reduces lethality, decreases cell adhesion, and enhances anti-phagocytic activity [48]. SSU0448 participates in N-acetylgalactosamine and galactosamine metabolism. Although no significant differences in pathogenicity were observed among mutant, wild-type, and complementation strains, Gram staining revealed reduced chain formation in the mutant strain, delaying SS2 pathogenesis, while chain formation was restored in the complementation strain. This indicates that SSU0448 regulates SS2 chain formation, indirectly affecting pathogenicity [49].

Recent studies suggest that at least nine multifunctional transcriptional regulators participate in SS2 virulence regulation, including five well-characterized factors—AdcR [50], CcpA [51, 52], ArgR [53], Rgg [54], and PerR [55]—plus four less-understood transcription factors: 00SSU005, treR, nadR, and scrR [56]. AdcR regulates zinc ion transport, and its expression blockage reduces bacterial virulence in mouse infection models [50]. Conversely, 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 exhibits reduced growth rate, decreased colonization capacity, attenuated virulence in mouse pathogenicity tests, significantly thinner capsule, and increased sensitivity to killing by porcine PMNs, confirming that ccpA is associated with SS2 virulence [33, 51, 52]. ArgR is a member of the arginine repressor family recently shown to regulate expression of the arcABC operon encoding arginine deiminase, thus considered a potential virulence factor [53, 58]. The Rgg regulator, identified in SS2, plays multifaceted 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 oxidative stress resistance protein (dpr) and methionine transporter (metQIN) [55].

These proteins and regulatory factors directly or indirectly participate in SS2 pathogenicity during infection, though their specific mechanisms of action require further investigation.

6. Conclusions and Outlook

Streptococcus suis serotype 2 poses a serious threat to the swine breeding industry and human public health, with prevention and control remaining challenging. Numerous scholars have conducted extensive research on SS2 virulence factors, with continuous reports of new putative virulence factors in recent years. However, studies on the contribution of these factors to bacterial pathogenicity have revealed that many are not primary virulence factors in the true sense, and their presence does not necessarily determine pathogenicity strength. This suggests that SS2 may possess additional unknown virulence factors, and pathogenicity may result from the combined action of multiple virulence factors with potentially complementary relationships, wherein partial functions of one factor may be compensated by another. Consequently, although the academic community recognizes distinctions between highly virulent, weakly virulent, and avirulent SS2 strains, no unified standard for distinguishing pathogenic from non-pathogenic strains has been established, and no definitive marker virulence factors have been identified to differentiate virulent, weakly virulent, and avirulent strains.

Research on established SS2 virulence factors indicates these factors may function in several aspects: anti-phagocytosis and protection from lysozyme killing [6, 12, 24-26, 28, 59]; adhesion facilitation [9, 23, 29, 32, 60]; stimulation of inflammatory cytokine production [6, 59, 61]; and participation in bacterial material and energy metabolism [1-3, 10, 11, 13, 14-17, 33]. However, many scholars now propose that these proteins may not be virulence factors per se, but their synthesis correlates with virulence [62]. Furthermore, do additional undiscovered virulence factors exist in SS2? Therefore, future research should focus on elucidating the mechanisms of individual virulence factors, their interrelationships, and the exploration and discovery of novel virulence factors. Recent work on virulence factors provides important references for identifying new protective antigens, investigating biological functions of new attenuated live

vaccines, establishing foundations for identifying new antigens closely associated with virulence, promoting deeper understanding of SS2 pathogenic mechanisms, enhancing comprehension of virulence factor pathogenicity, and providing theoretical guidance for screening new therapeutic drugs and designing vaccines to facilitate scientific and rational disease prevention and control strategies.

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