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EXTRACELLULAR VIRULENCE FACTORS OF GROUP B STREPTOCOCCI

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TABLE OF CONTENTS

1. ABSTRACT

Group B *Streptococcus* (GBS) is the leading cause of severe bacterial infections in human newborn infants. Advances in streptococcal molecular genetics and refinement of *in vitro* and *in vivo* model systems of GBS disease have led to the discovery and characterization of several extracellular virulence factors elaborated by this pathogen. This review summarizes our current understanding of GBS extracellular virulence factors including the beta-hemolysin/cytolysin, C5a-peptidase, hyaluronate lyase, CAMP factor, oligopeptidase, and carbohydrate exotoxin CM101. The molecular basis and potential pathogenic role(s) of each factor are considered in the context of neonatal infection.

2. INTRODUCTION

Group B *Streptococcus* (GBS) is the leading cause of invasive diseases in human newborns including pneumonia, sepsis and meningitis. GBS colonizes the lower gastrointestinal tract and vaginal epithelium of healthy adults, and infants can acquire GBS from the mother through aspiration of infected amniotic fluid or during passage through the birth canal. The more common "early-onset" form of GBS disease is an often fulminant infection developing shortly after birth, usually with clinical features of pneumonia and septicemia. Premature, low-birth weight infants are at greatly increased risk of early-onset GBS infection. "Late-onset" GBS infection can occur in infants from several days to several months of age, usually with a more subacute presentation and a high incidence of meningeal involvement. In recent years, the number of invasive GBS infections reported in adults has increased to levels comparable to the newborn population (1, 2); elderly and immunocompromised individuals are especially vulnerable.

The spectrum of GBS disease manifestations reflects a complex interplay of the host innate and adaptive immune systems with many surface-associated and secreted bacterial components. The special circumstances of vertical transmission and unique immune susceptibilities of the human newborn add further wrinkles and provide additional clues to those GBS factors that are most important in pathogenesis. Increasingly detailed knowledge of these virulence factors and the interacting immune components is providing a richer understanding of the disease process and revealing potential targets for improved therapy and prevention.

The pathogenesis of neonatal GBS infection is complex and multifactorial. First and foremost, the ability of GBS to effectively compete with other microflora and adhere to vaginal epithelial cells at low pH places the organism in position for transmission to the susceptible infant. While the large majority of infants exposed to GBS at delivery do not develop symptomatic disease, the organism possesses a number of phenotypic attributes that can result in invasive infection when newborn defense mechanisms fail. These GBS virulence traits include (a) factors that impede or subvert immunological clearance; (b) an ability to penetrate epithelial and endothelial cellular barriers to reach the bloodstream and deeper tissues; (c) toxins that directly injure or disrupt host tissue components; and (d) components that provoke inflammatory pathways which themselves may contribute to disease symptomatology. These bacterial factors are then considered in the context of a newborn host that possesses quantitative and qualitative deficiencies in phagocytes, specific antibodies, and complement components of the immune system. Such deficiencies are exaggerated when the infant is born prematurely or the bacterial challenge is

Table 1. Features of the Extracellular Virulence Factors of Group B *Streptococcu*s

Virulence factor	Genetic basis	Biochemical nature	Molecular or cellular actions	Proposed contribution(s) to disease pathogenesis	*In vivo* studies supporting a virulence role?
Beta-hemolysin/ Cytolysin	*cyl*E	CylE protein (87 kD)	forms pores in cell membranes induces apoptosis promotes cellular invasion triggers iNOS, cytokine release	direct tissue injury penetration of epithelial barriers induction of sepsis syndrome	Yes (Mice, Rats, Rabbits)
Hyaluronate lyase	*hyl*B	HylB enzyme (110 kD)	cleaves hyaluronan and chondroitin sulfate	spread through host tissues	No
C5a peptidase	*scp*B	ScpB protein (120 kD)	cleaves human C5a binds fibronectin	inhibit PMN recruitment reduce opsonophagocytosis host cell attachment/invasion	Yes (Transgenic Mice)
CAMP factor	*cfb*	CAMP protein (24 kD)	CAMP reaction (co-hemolysin) Binds to Fc portion of IgG, IgM	? tissue injury ? impairment of antibody function	Yes (Mice, Rabbits)
Oligopeptidase	*pep*B	PepB peptidase (70 KD)	cleaves bioactive peptides and perhaps collagen	? promotion of tissue invasion	No
Carboydrate toxin CM101	unknown	mannan-like polysaccharide	unknown	induction of sepsis syndrome	Yes (Mice, Sheep)

magnified by placental penetration and replication in the amniotic fluid.

Many GBS virulence factors represent integral components of the bacterial surface. Foremost among these is the GBS surface polysaccharide capsule, which through its terminal sialic acid motif acts to impede complement deposition and opsonophagocytosis. The current review will focus on extracellular virulence factors of GBS; i.e. those factors that can exert a virulence function even when physically separated from the bacterial cell. Some GBS extracellular virulence factors are secreted directly into the medium; others may be associated with the bacterial surface but can be removed in an active form by proteolysis or binding to host cell components (table 1). The reader is referred to several recent comprehensive reviews of GBS pathogenesis for additional discussion of purely cell-associated GBS virulence factors (3-6).

3. EXTRACELLULAR VIRULENCE FACTORS

3.1. Beta-Hemolysin/Cytolysin

A hallmark phenotype of GBS in the clinical laboratory is the appearance of beta-hemolysis surrounding colonies grown on the surface blood agar plates. The GBS beta-hemolysin has been of particular interest to researchers for decades because of its ability to lyse not only red blood cells but a broad range of eukaryotic cell types (7-9). Consequently, this toxin is more appropriately referred as a beta-hemolysin/cytolysin (beta-h/c). GBS beta-h/c activity is normally associated with the bacterial cell surface (10) and not present in abundance in the supernatant. However, GBS hemolysin activity can be extracted into the supernatant if a large carrier (or "stablizer") molecule such as albumin, starch, or lipotechoic acid is in the medium (11-13). Attempts to purify this molecule to homogeneity have been frustrated because cytolytic activity is rapidly lost when detached from the carrier. Consequently, little is known about the chemical structure of the active GBS beta-h/c. Stabilized GBS beta-h/c is inactivated by proteases such as subtilin, indicating the toxin is a protein (as are all other known pore-forming bacterial hemolysins). The GBS beta-h/c phenotype is invariably associated with an orange pigment of unknown function (9, 14, 15), and both are constitutively expressed, though their levels of expression are differentially regulated by changes in environmental conditions such as glucose and pH (14).

In the past decade, the advent of novel molecular tools has allowed investigators to elucidate the genetic basis for GBS beta-h/c production. The chromosomal locus encoding GBS beta-h/c activity was first discovered by analysis of nonhemolytic (NH) mutants generated using a novel transposition vector (16). The locus was designated *cyl* and included several open reading frames (ORFs) with homology to enzymes involved in fatty acid biosynthesis. Subsequent investigations by Pritzlaff *et al.* (17) adopted a positive selection approach wherein a plasmid library of the GBS chromosome was expressed in *E. coli* and a transformant identified that expressed beta-hemolysis. These studies independently identified and extended the GBS *cyl* locus to an operon of 12 genes. Subsequent fine mapping identified the *cylE* ORF, encoding a predicted 78 kDa protein without GeneBank homologies, as the structural gene for GBS beta-h/c activity. In frame-allelic exchange of the *cylE* gene ablated beta-h/c production in several GBS strains and complementation analysis of these mutants with a plasmid encoding *cylE* restored the wild-type phenotype. Heterologous expression of *cylE* alone in *E. coli* confers beta-hemolysis on blood agar plates. Thus the *cylE* ORF is both necessary and sufficient for GBS beta-h/c activity (17).

In vivo studies using isogenic GBS mutants with nonhemolytic (NH) or hyperhemolytic (HH) phenotypes have confirmed a role for this toxin in the pathogenesis of invasive infection. The degree of beta-h/c production correlates to GBS virulence as determined by LD_{50} in mouse or neonatal rat models of pneumonia (18, 19), a mouse model of bacteremia and arthritis (20), and a rabbit model of septicemia (21). Histopathologic examination shows that the degree of necrotic tissue damage is increased in HH mutants, and decreased or absent in NH mutants, when compared to wild-type GBS strains (20, 21). It is logical that direct cytolytic properties of the GBS beta-h/c toward host tissue cells contribute to virulence. In tissue culture studies, beta-h/c has been shown to damage fibroblasts (8), lung epithelial cells (9), lung endothelial cells (22), brain endothelial cells (23) and macrophages

(24). Injured cells showed surface bleb formation, dramatic loss of cytoplasmic density, splitting of the cytoplasmic and nuclear membranes, dilated organelles, and clumping of nuclear chromatin, all consistent with a pore-forming mechanism of action (9). Efflux kinetic studies indicate the beta-h/c toxin forms membrane lesions of large size (7).

In addition to direct cytotoxicity, the GBS beta-h/c exhibits proinflammatory, proapoptotic, and proinvasive properties that could contribute to disease pathogenesis. In the mouse, beta-h/c production is associated with increased local and systemic levels of proinflammatory cytokines (IL-6, IL-1alpha) while in the rabbit, beta-h/c-mediated liver injury is characterized by widespread hepatocyte apoptosis. *In vitro* studies confirm that the GBS beta-h/c upregulates macrophage expression of iNOS and nitric oxide (24), lung epithelial expression of IL-8 (25), and blood-brain barrier expression of a wide range of neutrophil signaling factors (e.g, IL-8, GRO-alpha, ICAM-1, GM-CSF) (26). GBS beta-h/c triggers apoptosis of macrophages *in vitro*, a process which is dependent on protein synthesis, but independent of caspase-1, caspase-3 or MyD88 signal tranduction pathways (27, 28). Beta-h/c further promotes the ability of GBS to invade pulmonary epithelial cells, a proposed early step in the pathogenesis of systemic infection (25).

The cytolytic properties of the GBS beta-h/c are inhibited in a dose-dependent fashion by phospholipids such as dipalmotyl phosphatidylcholine (DPPC), the major component of pulmonary surfactant (8, 9). DPPC can also inhibit GBS mediated cytokine activation (25, 29), macrophage apoptosis (27) and epithelial cell invasion (25). It is interesting to speculate that a lack of DPPC inhibition of beta-h/c toxicity may in part explain the increased incidence and severity of GBS pneumonia and sepsis in premature, surfactant-deficient neonates (4). In the future, adjunctive therapies designed to neutralize beta-h/c could prove of use in management of neonatal GBS infections; indeed, surfactant therapy appears to exert a beneficial effect against pneumonia and lung injury produced by the organism (30). Neutralizing antibodies against the GBS beta-h/c do not develop as a consequence of natural infection nor have they been raised successfully to crude GBS beta-h/c preparations (31). It is hoped that production of antibody to the recombinant CylE protein in a native or denatured form may prove to be a successful alternative.

3.2. Hyaluronate lyase

Hyaluronate lyase is part of a large family of enzymes called hyaluronidases, found in many Gram-positive pathogens. These enzymes are postulated to facilitate spread of bacteria by breaking down the hyaluronan polymers ubiquitously present in the extracellular matrices of the host. Hyaluronan is composed of repeating units of D-glucuronic acid (1-beta-3) N-acetyl-D-glucosamine (1-beta-4) (32), and cleavage by hyaluronate lyase occurs at the glycosidic bond between these two residues. A secondary substrate of hyaluronate lyase is chondroitin sulfate, which is involved in cushioning of surrounding structures and macromolecule transport. Cleavage of chondroitin sulfate could also be hypothesized to facilitate deep tissue penetration during infection. (33, 34).

The GBS hyaluronate lyase is encoded by the gene *hylB* and is expressed as a 110 Kd secreted protein (35, 36). This enzyme was initially misidentified as a neuraminidase because it was routinely used with bovine mucin in an assay to measure sialic acid release. Pritchard, on careful studies, identified that the compound released in the assay is actually a derivative of hyaluronic acid contaminating the mucin preparation (37). On encountering hyaluronan, GBS hyaluronate lyase makes random cuts in the substrate chain and releases disaccharides as it proceeds along the molecule (38). Details of this enzymatic activity were predicted based on the crystal structures of GBS hyaluronate lyase, and their complexes with both a disaccharide product of degradation, and a hexasaccharide unit of hyaluronan (39, 40). These studies showed that the lyase is composed of two domains, an alpha-helical alpha-domain and a beta-sheet beta-domain. Dynamics analyses suggest a mechanism by which the substrate chain is processed. First, a rotation of the alpha-domain in relation to the beta-sheet facilitates repositioning of the substrate so that it is ready for further cleavage at the catalytic site cleft. Movement of the two domains relative to each other also leads to a change in electrostatic characteristics which may allow binding to the negatively charged hyaluronan ligand. Finally, opening and closing of the substrate binding site brings the beta-1-4 glycosidic bond into close proximity of the catalytic histidine to facilitate cleavage of the bond.

The placenta and lung are especially rich in hyaluronan, and may represent preferential targets during the early stages in the pathogenesis of vertical transmission and early-onset infection. CD44, a ligand for hyaluronan, can be found on the surface of T and B lymphocytes, neutrophils and macrophages, and may be involved in extravasation of immune cells to sites of tissue infection and immune activation (41, 42). Thus it is conceivable that in addition to extracellular matrix disruption, hyaluronate lyase may play a role in interference of immune recruitment.

Consistent with the hypothesis that hyaluronate lyase is important in infection, it has been reported that the protease is expressed at higher concentrations in GBS isolated from neonates with bloodstream infection compared to GBS from asymptomatically colonized infants (43). Additionally, in a piglet model of pneumonia, GBS infection resulted in a significant decrease in the level of pulmonary hyaluronan (44). The precise role of the enzyme in pathogenesis still awaits confirmation through comparative analysis of the virulence potential of GBS wild-type strains and isogenic hyaluronate lyase-deficient mutants.

3.3. C5a Peptidase

During pulmonary infection, an expected host immune response is the activation of the complement cascade with breakdown of C5 produced by alveolar epithelial cells (45). C5a, a 74 amino acid cleavage product of C5, subsequently recruits neutrophils to the site of

infection (46). In fulminant early-onset GBS disease, the lungs often do not show the same degree of neutrophil influx seen in other pyogenic bacterial pneumonias (47, 48), a phenomenon commonly attributed to the immaturity of neonatal immune responses. Consistent with this hypothesis, neutrophils in newborns have been shown to be less capable of directed motility towards chemoattractants compared to their adult counterparts (49).

Through elegant work by Hill and coworkers, a GBS factor was identified that contributed to the delayed neutrophil response in neonatal lungs (50). Upon incubating activated C5a in the presence of GBS, they noted a significant depression in the level of chemotactic activity. SDS-PAGE analysis revealed degradation of the C5a molecule through loss of a 650 d fragment. The site of enzymatic cleavage was subsequently shown to be between histidine-67 and lysine-68 near the C-terminus of C5a (51), which is in close proximity to the active site of the C5a molecule (52).

The GBS C5a-peptidase is a 120 Kd protein encoded by the *scpB* gene (53). Similar peptidases are produced by groups A and G streptococci, and the high homology (95-98% identity) shared by the enzymes suggests a common ancestry or horizontal gene transfer (53-55). C5a-peptidase has a proteolytic domain at the N-terminal sequence and a peptidoglycan anchor on the C-terminal side. The enzyme is classified as a serine esterase based on its sensitivity to the inhibitor di-isopropyl fluorophosphate (56). Though normally displayed on the cell surface, active C5a-peptidase may be released from the bacteria following processing of the cell wall (56). In adult serum, the soluble form of C5a peptidase is neutralized by naturally occurring IgG, whereas the cell-bound form is protected from IgG by the capsule without hindering its peptidase function (57).

Analyses of the GBS genome have revealed two additional loci that potentially encode cell wall-bound serine proteases with 55% and 49% similarity to C5a peptidase. These putative proteases possess the identical catalytic domain of C5a peptidase, but the functional relevance of the genomic analysis has yet to be tested (58).

C5a peptidase may contribute to GBS pathogenesis in a variety of fashions. First, inactivation of C5a leads to attenuation of neutrophil chemotaxis in vitro (50). Efforts to validate this finding in vivo were initially hampered by the fact that the peptidase has minimal effect on C5a produced by rats, mice, pigs, rabbits, and sheep and is only active against human, monkey, and bovine C5a (59). Consequently, Bohnsack and colleagues resorted to the use of knockout mice deficient in C5a that have been reconstituted with human C5a. As expected, reconstitution with human C5a resulted in increased neutrophil influx into the lung following GBS infection. Challenge of the transgenic mice with a C5a-peptidase-deficient GBS mutant was associated with significantly more pulmonary neutrophil recruitment than observed in animals challenged with the wild-type bacteria (60).

In addition to neutrophil recruitment, C5a also enhances the opsonophagocytic capacity of neutrophils by upregulating their expression of surface complement receptor 3 (CR3) (61). *In vitro*, GBS reduced the opsonophagocytic property of neutrophils combined with C5a, presumably by interfering with C5a-induced upregulation of CR3.

Finally, it has long been noted that the 120 Kd protein is unusually large to simply code for a peptidase function. Recently, two groups have independently reported that C5a peptidase has an affinity for fibronectin (62, 63). The binding occurs independently of an arginine-glycine-aspartic acid (RGD) motif sometimes associated with extracellular matrix protein binding in bacteria (64). GBS intracellular invasion of lung epithelial cells can be abolished by addition of anti-C5a peptidase antibodies, suggesting that the cell-bound form of the molecule may also serve as an invasin (62).

Because the C5a-peptidase is a highly conserved molecule expressed by GBS from all capsular serotypes, it has been a focus of GBS vaccine development. Studies have shown that preincubation of GBS with antibod**ies** raised to C5a-peptidase enhances subsequent macrophage killing of the bacteria (65). Furthermore, immunization of mice with C5a-peptidase conjugated to capsular polysaccharide type III not only enhanced polysaccharide-specific antibody production, but also promoted serotype-independent killing of GBS as assessed by the number of bacteria recovered from lungs of infected animals (66).

3.4. CAMP factor

The CAMP reaction is eponymously named for its original descriptors: Christie, Atkins, and Munch-Peterson (67). This reaction refers to the synergistic hemolytic zones produced by colonies of GBS streaked adjacent to colonies of *Staphylococcus aureus* on sheep blood agar plates. In the literature, CAMP factor is better known for its utility in identification of GBS in the clinical laboratory, but there are data suggesting that it could also represent a virulence factor of the bacterium.

The CAMP factor was first purified to homogeneity by Bernheimer and coworkers (68). The co-hemolysin was found to be a 23.5 Kd extracellular protein with homologies to protein A of *S. aureus* (69). The gene encoding CAMP factor, *cfb*, has been subsequently cloned by two groups and the expressed recombinant protein was found to elicit antibody responses that blocked the CAMP reaction (70, 71). The *cfb* gene is not unique to GBS, as it is found in the genomes of a number of streptococcal species (72).

GBS CAMP protein has been shown to carry out two functions. In the CAMP reaction, the toxin damages sheep red cells presensitized to *S. aureus* sphingomyelinase (68). In a two step process, *S. aureus* sphingomyelinase treatment appears to enhance binding of the CAMP protein to the sheep red cell membrane, which is followed by a highly cooperative porin formation and subsequent cell lysis. Osmotic protection experiments using polyethelyne glycol and recombinant CAMP suggest that porins formed on the surface of sheep RBC are larger than 1.6 nm,

whereas pores observed on electron microscopy are 12-16 nm in diameter (73). CAMP protein also binds weakly to the Fc portion of human IgG and IgM (74).

In vivo studies suggest a potential contribution of CAMP protein to virulence. Skalka and colleagues produced mortality in rabbits and mice by direct injection of partially purified CAMP preparation (75). Jurgens *et al*, also working in the murine model, induced septicemia and death when CAMP was coadministered with a sublethal dose of GBS (74). It is unknown whether the cytolytic or immunoglobulin-binding properties of CAMP factor, or both, may contribute to disease pathogenesis.

3.5. Oligopeptidase

The presence of a "collagenase" in GBS was initially suggested by the presence of disordered amniotic collagen fibrils following infection of amnionitic tissue. The putative collagenase activity was isolated and shown to degrade a synthetic peptide N-(3[2-furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA), which mimics the primary structure of collagen (76, 77). Peptide-degrading activity was shown to be prevalent among a series of 25 clinical isolates of GBS, therefore a role for this enzyme in premature rupture of membrane was hypothesized. Further analysis of this activity showed that it does not meet the rigorous criteria of a collagenase, since the protein was unable to solubilize a film of reconstituted rat tail collagen (77). Following discovery of *pepB*, the gene encoding this activity, it was determined that the encoded protein possessed 66% identity to a PepF oligopeptidase from *Lactococcus lactis*, a member of the M3 or thimet family of zinc metallopeptidases. Testing of the collagenase-like enzyme against a panel of bioactive agents revealed that it is in fact an oligopeptidase capable of degrading a variety of small peptides, including bradykinin, neurotensin, and peptide fragments of substance P and adrenocorticotropin. Given these attributes, a theoretical role exists for this enzyme in pathogenesis through degradation of proteinaceous host defense factors.

3.6. Carbohydrate Toxin CM101

Extensive fractionation from large volumes of GBS culture supernatant has led to isolation of a noncapsular, high-molecular weight, mannan-like polysaccharide capable of inducing certain features of GBS sepsis symptomatology when injected into animals, including pulmonary hypertension, vascular permeability, and neutrophil trapping (78-80). The so-called carbohydrate toxin CM101 can be detected by ELISA in the spinal fluid and urine of infants with GBS sepsis (81). The molecular mechanisms by which this unusual compound can lead to host immune activation remain under investigation.

3.7. Extracellular virulence factors predicted from analyses of GBS genomic sequences

This past year has witnessed the publication of the complete genomic sequence of two wild-type GBS isolates (type III and V)(58, 82). Based on analyses of these genomes, more than 600 proteins are predicted to be membrane-associated or secreted, many of which have the potential to play a role in pathogenesis.

Both studies identified more than 20 putative proteins likely anchored to the cell wall through a C-terminal LPXTG motif. The LPXTG motif represents a common means by which adhesins and invasins anchor to the Gram-postive bacterial cell surface. Of the 30 putative proteins with this cell wall sorting signal motif within the genome reported by Glaser and coworkers, 13 had predicted functions. These include 3 peptidases, 2 nucleases, 1 amidase, 1 pullulanase, and 6 adhesins. Four of these have orthologs in *S. pyogenes*, 3 in *S. pneumoniae*, with the remaining 7 being unique to GBS.

Among the 71 secreted proteins predicted from the type III GBS genome, a neuraminidase-like protein was identified with significant homology to NanA of S. pneumoniae. In S. pneumoniae, neuraminidase has been shown to cleave the terminal sialic acid from cell surface glycans such as mucin, glycolypids, and glycoproteins, which leads to changes in the glycosylation patterns of the host and probably exposes more of the host cell surface. This is thought to contribute to increased adhesion, enhanced bacterial colonization and persistence in the nasopharynx and middle ears (83). Another interesting secreted protein identified in the genome is the fibronectin-binding protein that shared homology to the PavA gene of S. pneumoniae. This is an anchorless protein that is thought to bind to fibronectin after secretion from the bacteria and reassociation with the bacterial cell surface. In a murine model of sepsis induced by S. pneumoniae, the PavA deficient bacteria were found to be 104 fold attenuated in virulence (84, 85). Further consideration of the potential virulence role of the homologous proteins in GBS is anticipated.

4. PERSPECTIVE

Recent research efforts have identified a number of extracellular virulence factors that contribute to the pathogenesis of neonatal GBS infection. These studies have been performed with purified extracellular components of GBS and/or mutants failing to express the component in question. These GBS factors contribute to virulence by direct cytolytic injury to host tissues, disruption of extracellular matrix components, promotion of cellular invasion, impairment of neutrophil recruitment or phagocytosis, or activation of host inflammatory factors in the sepsis cascade (figure 1).

Our understanding of the precise molecular mechanisms by which each GBS factor contributes to disease pathogenesis will be furthered by further refinement of techniques for nonpolar targeted mutagenenesis or controlled expression of individual genes and their encoded gene products. Novel sophisticated molecular analyses such as signature-tagged mutagenesis (86), phage-display (63) or proteomics (87) are revealing further candidate GBS virulence determinants.

Finally, the publication of the complete genomic sequence of the two wild-type GBS isolates (type III and

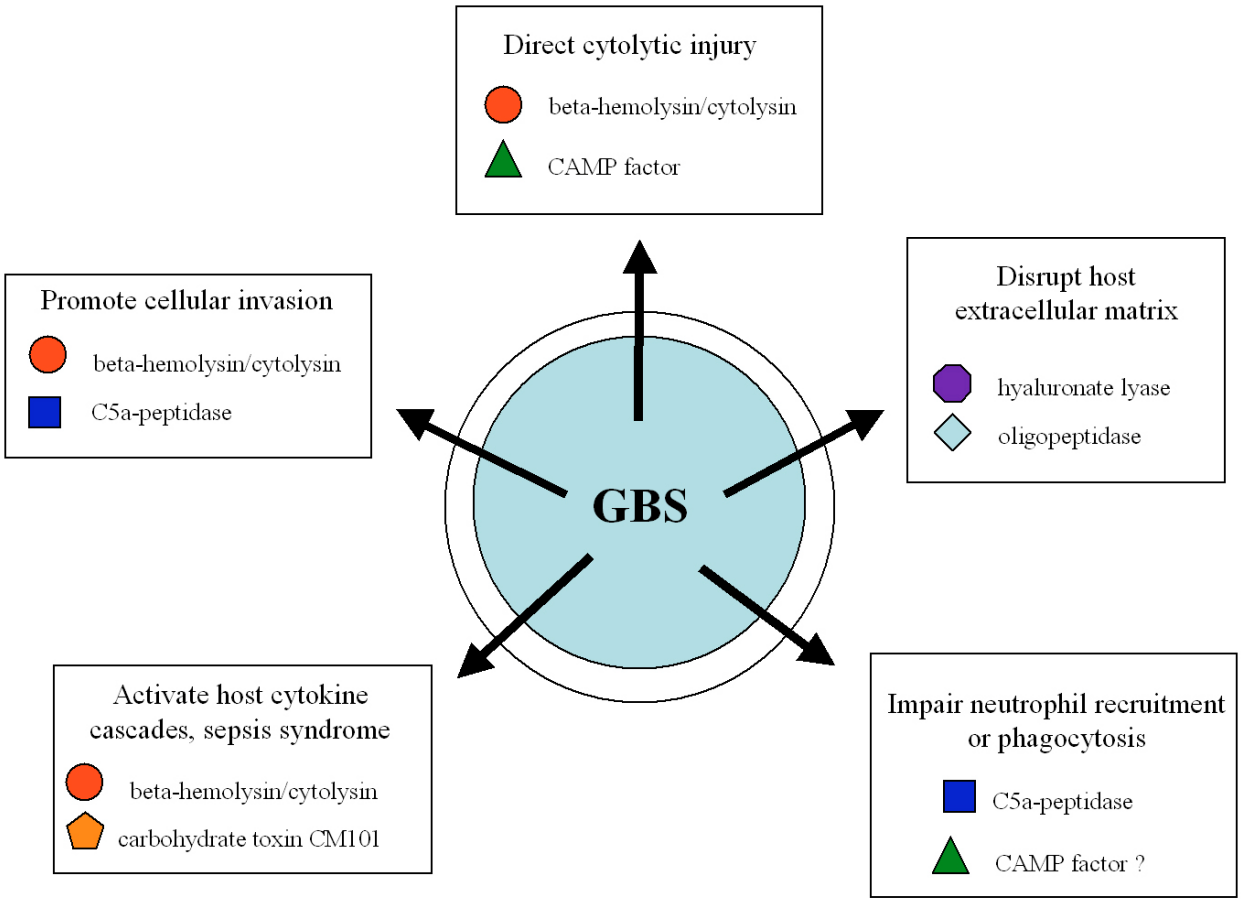

Figure 1. Roles in pathogenesis played by GBS extracellular virulence factors.

V) provides an invaluable new resource for the study of the molecular pathogenesis of these organisms (58, 82).These databases will facilitate analysis of additional GBS genes that share homology to known extracellular virulence factors of related bacterial species. Together, these molecular approaches to analyzing GBS virulence mechanisms promise to inform the design of novel therapeutic and preventive measures to combat this foremost of newborn pathogens.

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