CHAPTER

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The group B streptococcal β-hemolysin/cytolysin

George Y. Liu and Victor Nizet

INTRODUCTION

Group B *Streptococcus* (GBS) is the leading cause of invasive bacterial infections in human newborns, and is increasingly recognized as a pathogen in adult populations including the elderly, pregnant women, and diabetics (Berner, 2004; Blancas *et al*., 2004). The GBS β-hemolysin/cytolysin (βH/C) is a surface-associated toxin that plays an important role in virulence of the organism. The βH/C is also responsible for the characteristic zone of clearing around GBS colonies grown on blood agar plates, a useful diagnostic phenotype in the clinical microbiology laboratory.

The GBS βH/C was recognized as early as the 1930s (Todd, 1934), but was largely neglected from an experimental standpoint. Nearly a half century later, the emergence of GBS invasive disease in newborns triggered renewed interest in the toxin as a potential virulence factor of the pathogen. Studies in the early 1980s provided insight on the basic biochemical properties of the βH/C and its mechanism of erythrocyte lysis. However, further in-depth analysis was hindered by the toxin's lability and apparent non-immunogenicity, factors that to this day pose great challenges to final purification. In the 1990s, the advent of molecular genetic tools for GBS analysis allowed generations of isogenic mutant strains with altered βH/C phenotypes. With this technological breakthrough, the role of βH/C in GBS disease has been explored extensively in tissue culture and small animal models of infection. These studies have shown a toxin with a broad array of cytolytic, proinvasive, proapoptotic, and proinflammatory properties that contribute in a significant fashion to the virulence of GBS (Nizet, 2002; Liu *et al*., 2004). Current knowledge thus suggests the βH/C may represent an attractive therapeutic target in management of neonatal GBS disease.

BIOCHEMISTRY AND GENETICS

Basic properties of βH/C

The first report of the GBS βH/C provided by Todd (1934) described an extracellular molecule that is oxygen stable, acid and heat labile, and non-immunogenic. Analogies were drawn to streptolysin S (SLS) of *group A Streptococcus* (GAS), because the two β-hemolysins share many properties at first glance, including their inherent lability, broad-spectrum cytolytic activity, and lack of immunogenicity (Ginsburg, 1970). However, genetic evidence to be discussed below has ultimately revealed these toxins to be unrelated molecules (Nizet, 2002).

Biophysical properties of the GBS βH/C have placed technical hurdles in the path of its purification. The molecule is acid and heat labile, with significant loss of activity at 50°C after 15 minutes (Dal and Monteil, 1983). Normally associated with the bacterial cell surface, GBS βH/C activity is facilitated through direct contact between the bacterium and the target eukaryotic cell (Platt, 1995). Simple culture supernatants of GBS do not possess measurable βH/C activity. However, the presence of certain high-molecular weight "carrier" molecules in solution can stabilize extracellular toxin activity (Ginsburg, 1970).

As experimental proof, placement of an 0.22 micron filter between the GBS and the target erythrocyte blocks hemolysis unless a carrier molecule is added to the medium (Platt, 1995). Examples of carrier molecules that have shown an ability to stabilize βH/C activity include serum proteins such as albumin, Tween 80 and related detergents, starch polymers, and lipotechoic acid (Marchlewicz and Duncan, 1980; Ferrieri, 1982; Tsaihong and Wennerstrom, 1983). In attempted protocols for βH/C purification, separation of the candidate toxin from its carrier molecule has invariably been associated with loss of hemolytic activity.

In an early report on partial purification of the GBS βH/C activity (Marchlewicz and Duncan, 1980), researchers harvested GBS in late log growth phase, resuspended the bacteria in phosphate buffered saline supplemented with 1% glucose plus the stabilizers Tween 80 and starch, and then concentrated the supernatant by methanol or ammonium chloride precipitation. When the extract was applied to a Sephacryl S-300 column, two distinct peaks were noted, one of which harbored most of the hemolytic activity. However, electrophoretic separation of this peak revealed the presence of numerous protein bands. In a subsequent published protocol, βH/C was extracted from a GBS pellet by sonication in a buffer containing bovine serum albumin (BSA) and further purified by gel filtration (Dal and Monteil, 1983). Eluent from the column also produced two peaks, the first of which, on electrophoresis resolved into four protein bands. The top three bands were nonhemolytic and consisted of BSA polymer, dimer, and monomer. The front running band was hemolytic, but harbored traces of BSA. The authors attempted further purification by agarose gel electrophoresis or affinity chromatography on protein-absorbing Blue Sepharose CL6B, but these techniques failed to separate hemolytic activity from the BSA carrier.

The above observation of a hemolytic front running band smaller than the size of BSA monomer (about 67 kD) appears to place an upper limit on the size of the mature toxin (Dal and Monteil, 1983). Moreover, during efforts to purify the βH/C following extraction of GBS with detergent or lipoteichoic acid (LTA), it was observed that βH/C can be bound to these macromolecules without significantly affecting their respective elution profiles macromolecule on column chromatography, suggesting that the βH/C is likely to be a relatively small molecule (Tsaihong and Wennerstrom, 1983).

GBS βH/C expression occurs primarily between the early exponential and early stationary phase of growth (Platt, 1995). In a GBS chemostat system where βH/C is constantly extracted with Tween 40 in the growth medium, optimal release was measured during the late exponential phase. βH/C production also increases significantly under experimental conditions of enhanced GBS growth rate (Ross *et al*., 1999). Production of the toxin is greatly enhanced if a low concentration of glucose (about 2 mg/ml) or other fermentable carbohydrates is present in the medium, but unexpectedly, glucose in the range of 10 mg/ml almost entirely abolished βH/C production, pointing to regulatory linkages of βH/C production to cellular metabolism (Tapsall, 1986). Consistent with this hypothesis, treatment with various metabolic including sodium fluoride, iodoacetate, or 2-deoxy-glucose greatly reduced the production of assayable βH/C.

Mechanism of βH/C pore formation

It is notable that GBS βH/C is cytolytic for a very broad range of eukaryotic cells, including erythrocytes, fibroblasts (Tapsall and Phillips, 1991), lung epithelial and endothelial cells (Nizet *et al*., 1996; Gibson *et al*., 1999), brain endothelial cells (Nizet *et al*., 1997b), and macrophages and neutrophils (Liu *et al*., 2004). By implication, the βH/C must interact with and damage molecular targets common to the cell membranes of all these cells. Though these host molecules have not been fully defined, it is notable that βH/C activity can be markedly inhibited by phospholipids, such as dipalmitolyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylethanolamine (DPPE) (Tapsall and Phillips, 1991). This finding suggests that the GBS βH/C may possess an affinity for similar phospholipids in the eukaryotic cell membrane bilayer and that this affinity could guide the toxin to its site of action. In contrast with streptolysin O (SLO) of GAS and related cytolytic toxins, cholesterol does not inhibit GBS βH/C activity.

Membrane pore formation is thought to represent a principal mechanism by which the GBS βH/C exerts its toxic activity, and this phenomenon has been most closely examined in studies of erythrocyte lysis. Marchlewicz and Duncan showed that upon incubation of GBS βH/C extract with a 1% suspension of erythrocytes at 37°C, sufficient toxin associated with the target cells to produce 80% lysis within three minutes (Marchlewicz and Duncan, 1981). βH/C binding to erythrocytes appeared irreversible, unlike the interactions observed with SLS of GAS (Duncan and Mason, 1976). Also in marked contrast to SLS action, GBS βH/C exhibits a relatively short prelytic lag period, instead resembling lysis by GAS SLO or *Clostridium perfringens* theta-toxin (Bernheimer, 1947). Kinetic analysis revealed that rubidium ion and hemoglobin are released at the same rate from erythrocytes treated

with GBS βH/C, implying that the toxin creates membrane pores of relatively large size (Marchlewicz and Duncan, 1981).

Binding to erythrocytes by the βH/C, the length of the prelytic lag period, and the rate of hemolysis were found to be temperature dependent (Marchlewicz and Duncan, 1981). Since no toxin binding was noted at 0°C, it was proposed that a temperature-dependent fluidity of the membrane is necessary for GBS βH/C adsorption to the erythrocytes. As the incubation temperature increased, the length of the prelytic period decreased and the rate of lysis increased. Using an assay in which a fixed concentration of βH/C is incubated with an increasing concentrations of erythrocytes, the authors determined that more than one βH/C molecule is likely required to lyse a single cell (Marchlewicz and Duncan, 1981). When the logarithm of the fraction of unlysed RBC was plotted against βH/C concentration, a multi-hit survival curve was obtained (Atwood and Norman, 1949). Prediction based on this graph suggests that five molecules of βH/C are required to lyse a single sheep erythrocyte, reflecting a potency identical to that predicted for SLO of GAS (Inoue *et al*., 1976).

Genetic basis of βH/C production

The first major advance toward understanding the genetics underlying GBS βH/C activity was achieved by Spellerberg and coworkers through negative selection (Spellerberg *et al*., 1999). Using a novel pGh9:ISS1 transposition vector, the group generated a mutant library from which they identifed several nonhemolytic GBS mutants. The transposon insertion sites all clustered within a seven kilobase genetic locus of the GBS chromosome that was named *cyl*, in reference to the cytolytic activity exhibited by the βH/C (Figure 43.1). This study and subsequent reports of the GBS *cyl* gene cluster identified 12 separate open reading frames (ORFs) organized in an operon structure (Spellerberg *et al*., 1999; Spellerberg *et al*., 2000; Pritzlaff *et al*., 2001). The deduced amino acid sequences of four of the genes (*cylD*, *cylG*, *acpC*, *cylZ, cylI*) demonstrate significant homologies to enzymes of prokaryotic fatty acid biosynthesis. Furthermore, the clustered arrangement of these genes in proximity to an acyl carrier protein (*acpC*) gene is reminiscent of the *fab* gene clusters of *Escherichiae coli, Vibrio harveyi*, and *Bacillus subtilis* (Rawlings and Cronan, 1992; Morbidoni *et al*., 1996; Shen and Byers, 1996). The predicted products of *cylA/B* share significant homologies to prokaryotic and eukaryotic ATP binding cassette transporters, which are known to be required for the hemolysin export in *E. coli* (Felmlee *et al*., 1985), *Bordetella pertussis* (Glaser *et al*., 1988), and *E. faecalis* (Gilmore *et al*., 1990).

The association of the *cyl* locus with GBS βH/C production was corroborated through independent positive screening of a plasmid library of GBS chromosomal DNA in *E. coli* with the aim of identifying hemolytic transformants (Pritzlaff *et al*., 2001). One such hemolytic clone was isolated and the purified plasmid found to contain the GBS *cyl*E and downstream *cyl*F ORFs, as well as parts of the adjacent ORFs *cyl*B and *cyl*I. Confirmed targeted plasmid integrational disruption of *cyl*B, *cyl*F, and *cyl*I in three different GBS strains failed to consistently abrogate βH/C production. In contrast, targeted disruption of *cyl*E invariably produced a non-hemolytic phenotype, suggesting that of these genes, only *cyl*E was essential for βH/C expression (Pritzlaff *et al*., 2001). Precise in-frame allelic replacement of *cyl*E with an antibiotic cassette yields a NH mutant (Figure 43.1) in which βH/C activity can be

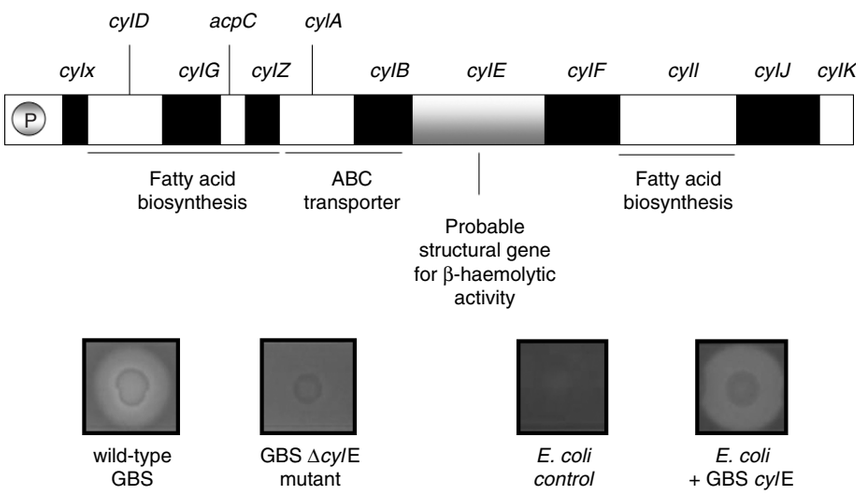

FIGURE 43.1 Map of the GBS *cyl* operon encoding β-haemolysin/cytolysin activity. Allelic replacement of the *cyl*E ORF yields a nonhemolytic mutant, while heterologous expression of *cyl*E in *E. coli* is sufficient to confer a β-hemolytic phenotype (Spellerberg *et al*. 1999; Pritzlaff *et al*. 2002).

restored by reintroduction of *cyl*E on a plasmid vector. When expressed in *E. coli* as a recombinant fusion protein, *cyl*E alone is sufficient to confer a robust β-hemolytic phenotype after 48 hours incubation (Pritzlaff *et al*., 2001) (Figure 43.1). The combination of the mutagenesis, complementation, and heterologous expression studies strongly suggests that *cyl*E encodes the structural determinant of the GBS βH/C, but does not exclude the possibility that additional GBS genes are involved in its processing, activation, or export to the cell surface. The predicted product of the *cyl*E ORF is a 78 kilodalton protein without significant homology to any other proteins in the GenBank databases. It remains to be determined whether the full length protein or a derivative represents the mature toxin, and the lack of sequence homologies precludes further inference on the precise mechanism of βH/C action at this time.

Interestingly, GBS exhibiting a hyperhemolytic (HH) phenotype can be generated by chemical or transposon mutagenesis (Weiser and Rubens, 1987; Nizet *et al*., 1996). These observations suggest the existence of regulatory pathways that may contribute to the observed variations in βH/C expression with growth phase or glucose concentration, for example. Recently, one clear negative regulator of GBS *cyl* operon expression was identified, a homologue of the *cov*S-*cov*R two-component global transcriptional regulator that is so well studied in GAS (Lamy *et al*., 2004). A GBS *cov*S/R knockout mutant showed markedly increased βH/C activity, and *cov*R was shown to bind directly to the promoter motif at the head of the *cyl* operon (Lamy *et al*., 2004).

Association with βH/C with pigment

An interesting feature of βH/C observed during biochemical and genetic studies is the close association of the βH/C to an orange pigment, also expressed on the surface of GBS. This pigment exhibits a triple peak absorbance at 455, 485, and 520 nm highly characteristic of β-carotene and related pigments (Merritt and Jacobs, 1978; Tapsall, 1986). The GBS carotenoid is quite labile and degrades readily to a single 425 nm peak when heated or subjected to freeze thawing. A close association with the βH/C was apparent because the pigment co-precipitated and co-purified with hemolytic activity (Tapsall, 1986). In fact, the inability to separate the two phenotypes led early investigators to conclude that the pigment could represent the natural carrier for the βH/C or even that the two molecules could be one *in the* same. However, published reports have since shown that the GBS βH/C and pigment are likely to be different molecules. For example, βH/C activity can be inactivated by subtilin, a serine protease. Other studies have shown that propagation of GBS under different pH and glucose concentrations results in differential yields of βH/C protein and pigment (Tapsall, 1987).

The close linkage between the GBS βH/C and carotenoid pigment holds true at the genetic level, since a screen of a transposon library for non-pigmented mutants once again mapped all insertions to the *cyl* locus (Spellerberg *et al*., 2000). Targeted mutagenesis of *cyl*E performed to knock out the βH/C phenotype also invariably knocked out expression of the orange pigment, and pigmentation returned when *cyl*E was used to complement the mutant *in trans* (Pritzlaff *et al*., 2001). Among clinical isolates, there is a direct correlation between the degree of pigmentation of the GBS colony and the strength of the surrounding zone of β-hemolysis (Merritt and Jacobs, 1978; Tapsall, 1986, 1987). And whether they were generated by chemical, transposon, or targeted mutagenesis, all NH GBS derivatives lack pigmentation and all HH derivatives display enhanced pigmentation (Nizet *et al*., 1996; Spellerberg *et al*., 1999; Spellerberg *et al*., 2000; Pritzlaff *et al*., 2001).

One should not conclude that one gene (*cyl*E) encodes both the βH/C and pigment since all known carotenoid pigments are assembled in a series of enzymatic steps. Neither of the published complete GBS genome sequences (Glaser *et al*., 2002; Tettelin *et al*., 2002) contain homologues of the enzymes typical of bacterial carotenogenesis (e.g., phytoene synthases, phytoene or carotene dehydrogenases, or lycopene cyclases); thus, the GBS pigment would appear to be the product of a unique biosynthetic pathway. The GBS pigment was recently found to play a role in pathogenesis by virtue of antioxidant properties consistent with the known oxygen radical scavenging potential of carotenoids. The pigment conferred protection to GBS against the antimicrobial effects of hydrogen peroxide, singlet oxygen, superoxide, and hypochlorite, all of which play a role in the oxidative burst-killing mechanism of host phagocytic cells (Liu *et al*., 2004). As a consequence, wild-type (WT) GBS has enhanced intracellular survival compared to non-pigmented, NH Δ*cyl*E mutants in neutrophil and macrophage killing assays, a difference which persists even when DPPC is added to block βH/C activity (Liu *et al*., 2004).

BIOLOGICAL EFFECTS

Cytolytic injury to eukaryotic cells

GBS βh/c has been demonstrated to lyse several different cell types, including sheep erythrocytes, murine

fibroblasts (Tapsall and Phillips, 1991), and human lung epithelial cells (Nizet *et al*., 1996; Doran *et al*., 2002), lung endothelial cells (Gibson *et al*., 1999), brain endothelial cells (Nizet *et al*., 1997b), and macrophages (Ring *et al*., 2000; Liu *et al*., 2004). Electron microscopic examination of GBS βh/c-hemolysin-induced injury to lung epithelial cells is consistent with the action of a pore-forming toxin (Figure 43.2). Discrete membrane disruptions, cellular swelling, loss of intracytoplasmic density, changes in subcellular organelles and chromatin are seen that appear to reflect entry of water into the cell and hypo-osmotic damage (Nizet *et al*., 1996). The GBS βh/c contributes to a loss of integrity in the tight junctions of polar lung endothelial cell monolayers, allowing passive flux of macromolecules across tight junctions (Gibson *et al*., 1999). There exist no reports to date of any eukaryotic cell types resistant to the cytotoxic effects of the GBS βh/c.

Triggering of cellular apopotosis

The clumping of nuclear chromatin observed in epithelial cells injured by GBS βh/c is reminiscent of the pattern observed with apoptosis or programmed cell death. Live GBS has been shown to induce apoptosis in macrophages (Fettucciari *et al*., 2000; Ulett *et al*., 2003; Liu *et al*., 2004), splenocytes (Liu *et al*., 2004) and hepatocytes (Ring *et al*., 2002a), and several investigators have explored a potential linkage of the βH/C to this form of induced cell death.

Fettucciari and co-workers described a hemolytic GBS strain that upon incubation with murine peritoneal macrophages, induced apoptosis of the host cells within 24 hr (Fettucciari *et al*., 2000). Membrane permeability changes were noted as early as 2 hr in 70% of cells by propidium iodide exclusion studies, and progression to apoptosis was demonstrated by electron microscopy, DNA fragmentation assay, and *in situ* TUNEL (terminal deoxynucleotide transferase-mediated dUTP nick end labeling) staining. Under conditions that either inhibited βH/C production (high glucose concentrations) or inhibited βH/C activity (addition of DPPC), apoptosis was abrogated. These observations led the authors to suggest that the βH/C was responsible for macrophage apoptosis. Subsequently, Ulett *et al*., compared the ability of WT or Δ*cyl*E mutant GBS to induce apoptosis in J774 macrophages (Ulett *et al*., 2003). Under their experimental conditions, the viability of macrophages exposed to each strain was similar, but significantly less than that of macrophages exposed to GBS that had been grown in high-glucose media. The authors proposed that macrophage apoptosis induced by GBS was independent of βH/C, and instead reflected the action of a GBS factor coregulated along with βH/C by glucose.

Our recent study attempted to shed light on these discrepancies by assessing macrophage apoptosis induced by GBS strains expressing different levels of βH/C and their corresponding NH Δ*cyl*E allelic exchange mutants (Liu *et al*., 2004). Apoptosis of murine peritoneal macrophage apoptosis was noted only with a strong hemolytic WT strain, but not with a weak hemolytic strain or either of the Δ*cyl*E mutants. The level of apoptosis was more prominent at higher bacterial inocula and could be inhibited with DPPC. Incubation of macrophages with an increasing dose of crude βH/C extract led to a dose-dependent increase

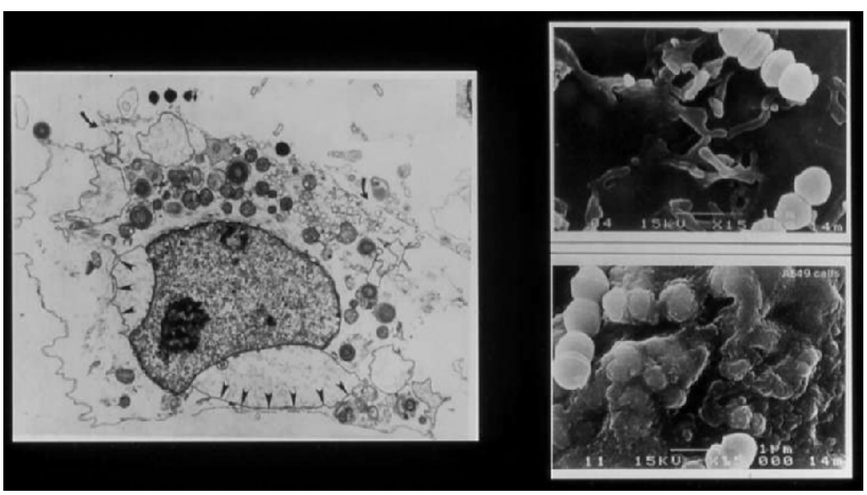

FIGURE 43.2 GBS β-hemolysin/cytolysin mediated injury to cultured human lung epithelial cells. At left, transmission electron microscopy shows membrane pore formation, swelling of cell contents, and clumping of nuclear chromatin. At right, the normal villous architecture of the lung epithelial surface (top) seen with a weakly hemolytic GBS isolate is abolished to swelling and bleb formation upon exposure to a strongly hemolytic isolate (below) (Nizet *et al*., 1996).

in the number of TUNEL positive-staining macrophages. In mice challenged intravenously with GBS, apoptosis was abundantly evident among splenocytes in those animals infected with a strongly hemolytic WT GBS strain but not in mice challenged with its isogenic Δ*cyl*E mutant (Liu *et al*., 2004). In a study of rabbits challenged intravenously with WT and βh/c mutant GBS, positive TUNEL staining and activation of caspase-3 in hepatocytes were also correlated to toxin production (Ring *et al*., 2002a). The balance of experimental data suggest that GBS induces apoptosis by βH/C-dependent and βH/C-independent mechanisms, with the toxin playing a greater role at higher bacterial concentrations and in the case of strongly hemolytic strains.

The mechanism of GBS βH/C-induced apoptosis may reflect subcytolytic perturbance of membrane integrity and influx of calcium ion, as the degree of apoptosis is predictably modulated through addition of excess cation to the media or chelation with EGTA (Fettucciari *et al*., 2000). More detailed analyses by Fettucciari and colleagues pointed to the involvement of c-Jun NH_2 terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), three members of mitogen-activated protein kinases (MAPKs) family, in GBS-induced macrophage apoptosis (Fettucciari *et al*., 2003). The authors demonstrated that during induction of apoptosis, WT hemolytic GBS stimulates a strong and persistent activation of JNK and p38 with concomitant inhibition of ERK. In contrast, use of a NH GBS strain induces only a transient activation of JNK, p38, and ERK MAPKs with no resultant apoptosis.

Cell activation and proinflammatory signaling

Membrane pore formation and resulting ion fluxes likely result in activation of signaling cascades within host cells. Pore formation may also facilitate injection of other effector molecules that directly activate certain host signaling pathways, as has been documented both in enterobactereciae (Cornelis and Van Gijsegem, 2000) and with streptolysin O of GAS (Madden *et al*., 2001). A pore-forming toxin of *Streptococcus pneumoniae*, pneumolysin, interacts with Toll-like receptor 4 (TLR-4) to stimulate innate inflammatory responses (Malley *et al*., 2003). Although it is unknown whether any or all of these potential mechanisms apply, the GBS βH/C has been shown to activate certain inflammatory response genes in target cells.

In human lung epithelial cells, the GBS βH/C stimulates gene transcription and release of interleukin-8 (IL-8), a potent neutrophil chemokine (Doran *et al*., 2002). The βH/C also promotes GBS intracellular invasion of lung epithelium, and both IL-8 release and invasion can be inhibited by the addition of DPPC (Doran *et al*., 2002). In human brain microvascular endothelial cells, oliognucleotide microarray analysis was employed to assess the transcriptional response of the blood-brain barrier to GBS and to specific GBS virulence factors (Doran *et al*., 2003). It was observed that GBS infection of the brain endothelium induced a highly specific and coordinate set of genes that act to orchestrate neutrophil recruitment and activation. These included the chemokines IL-8, Groα and Groβ, the neutrophil stimulating growth factor GM-CSF, the antiapoptotic factor Mcl-1, and the endothelial neutrophil receptor and activating molecular ICAM-1. Strikingly, infection of the endothelial cells with a Δβh/c mutant resulted in a marked reduction in expression of these genes involved in the neutrophil inflammatory response. Cell-free bacterial supernatants containing the βh/c toxin induced IL-8 release, identifying the toxin as the principal provocative factor for blood-brain barrier inflammatory activation. Migration of human neutrophils across polar brain endothelial cell monolayers was stimulated by the βh/c through a process involving IL-8 and ICAM-1 (Doran *et al*., 2003).

In murine macrophages, βH/C induces the expression of nitric oxide synthase (iNOS) and the release of NO, a potent vasodilator proximal mediator of the sepsis cascade (Ring *et al*., 2000). Stimulation of NO release by βH/C appears to depend on protein tyrosine kinases and NF-κB, suggesting the involvement of intracellular pathways similar to those that mediate lipopolysaccharide-induced iNOS activation (Ring *et al*., 2000). The induction of NO by GBS βH/C likely requires a coactivating factor be present on the cell surface, as a cell-free βH/C extract does not trigger NO production. In non-activated macrophages, synergistic activation by GBS cell wall components and βH/C is necessary to induce a NO response, which indicates that both factors cooperate to substitute for the priming signal typically provided by IFNγ (Ring *et al*., 2002b).

In summary, the GBS βH/C is a potent toxin with proven cytolytic activity against a wide variety of cell types encountered during the various steps of human disease pathogenesis. At subcytolytic doses, the GBS βH/C exerts a variety of proinvasive, proinflammatory, and proapoptotic effects on target cells. Therefore, it is logical to hypothesize that such effects would have deleterious consequences on the infected host. The contribution of the βH/C toxin to GBS pathogenesis has been investigated in several *in vivo* models of invasive disease and is reviewed in the next section.

βH/C IN THE PATHOGENESIS OF GBS NEONATAL INFECTION

Pneumonia, sepsis, and meningitis are the three most common clinical manifestations of invasive GBS infection in human newborns. In the more frequent instance of early-onset infection, the infant directly aspirates an inoculum of GBS from contaminated amniotic or vaginal fluids *in utero* or during passage through the birth canal. These infants present with respiratory signs indicative of an initial pulmonary focus of infection, and the GBS quickly spreads to the bloodstream to produce acute septicemia. Late-onset GBS infections can develop in infants up to several months of age, and are suspected to reflect mucosal colonization with the bacterium and subsequent invasion into the bloodstream. These infections often have a subacute presentation with a high rate of dissemination to end organs, such as the central nervous system to produce meningitis, or to skeletal foci to produce osteomyelitis or septic arthritis (Nizet and Rubens, 2000; Doran and Nizet, 2004). Because clear βH/C activity is apparent in the vast majority of GBS isolated from clinical sources, several efforts have been directed at identifying the role of this toxin in disease pathogenesis. The availability of isogenic transposon or allelic exchange mutants with altered βH/C phenotype has allowed direct assessment of the toxin in several small animal models of GBS infection.

Pneumonia

The first *in vivo* demonstration of a βH/C effect on lung pathology was accomplished using chemically-derived NH and HH GBS mutants (Wennerstrom *et al*., 1985). These researchers used intranasal inoculation to challenge mice with GBS, and found that increased βH/C activity was associated with greater degrees of cellular injury, as measured by lactate dehydrogenase release into the bronchalveolar fluid, and a decreased LD_{50} and earlier time to death for a given inoculum. Similar results were observed upon transthoracic challenge of neonatal rats with WT GBS and isogenic HH and NH transposon mutants. LD_{50} at 48 hr for the NH mutant was 1,000-fold greater than LD_{50} for either the WT strain or HH mutant, and the HH mutant lead to death of the infected animal more rapidly than the WT strain (Nizet *et al*., 1997a).

Most recently, pathophysiology associated with βH/C was investigated in a neonatal rabbit model of pneumonia induced by direct intracheal injection of GBS bacteria (Hensler *et al*., 2005). Animals infected with wild-type (WT) GBS developed focal pneumonia and by 18 h had 100-fold more bacteria in lung tissues than rabbits infected with the Δβh/c mutant. Mortality, development of bacteremia, and mean bacterial blood counts were all significantly higher in the animals challenged with WT GBS compared to the Δβh/c mutant. Lung compliance during mechanical ventilation was impaired upon infection with WT GBS but not with the Δβh/c strain. This work provided *in vivo* evidence for a critical role of the βh/c toxin in GBS neonatal pneumonia and the breakdown of the pulmonary barrier to systemic infection.

When a heavy inoculum of GBS is aspirated by the newborn, βH/C is likely to contribute to pneumonia by direct lysis of lung epithelial and pulmonary endothelial cells and perhaps alveolar macrophages, contributing to barrier breakdown and systemic dissemination (Nizet *et al*., 1996; Liu *et al*., 2004; Hensler *et al*., 2005). Supporting this hypothesis are the *in vitro* studies demonstrating a direct correlation of GBS βH/C activity to lung cellular injury as measured by LDH release, trypan blue nuclear staining, albumin flux, and ultrastructural examination (Nizet *et al*., 1996; Gibson *et al*., 1999). The stimulation of IL-8 release by the βh/c may contribute to neutrophilic inflammation seen in neonatal pneumonia, whereas the toxin's promotion of GBS cellular invasion may facilitate bloodstream entry (Doran *et al*., 2002). The very same phospholipid DPPC that inhibits βH/C-induced cytotoxicity (Nizet *et al*., 1996), apoptosis (Fettucciari *et al*., 2000; Liu *et al*., 2004), and proinflammatory effects (Doran *et al*., 2002) is also the major constituent of human pulmonary surfactant (70% by weight). Deficiency in pulmonary surfactant may explain the markedly increased risk of premature, low birth weight infants who suffer severe GBS pneumonia and sepsis (Tapsall and Phillips, 1991; Doran and Nizet, 2004).

Septicemia

Sepsis occurs when GBS successfully breach the primary barrier of the body to produce clinical symptoms of hemodynamic instability and organ dysfunction. The first evidence that the GBS βH/C could contribute to the pathogenesis of septicemia was provided by Griffiths and Rhee who found that systemic administration of a GBS βH/C extract to rabbits produced a significant acute hypotensive reaction and a limited number of deaths due to shock (Griffiths and Rhee, 1992). In parallel experiments, injection of GAS streptolysin S extract had no such effect on hemodynamics. In comparison of WT GBS and βH/C mutants in

murine intravenous challenge models of septicemia, the toxin is found to contribute markedly to an overall mortality and rapidity of death (Puliti *et al*., 2000; Liu *et al*., 2004). These changes are likely to reflect a combination of the antiphagocytic and proinflammatory properties of the toxin. The ability of GAS βh/c to lyse host neutrophils and macrophages and/or trigger their apoptosis leads to enhanced survival of the organism on encounter with the phagocytic cells (Liu *et al*., 2004). Systemic release of the proinflammatory cytokine IL-6 is greatly increased in response to a hemolytic GBS strain compared to its isogenic NH mutant (Puliti *et al*., 2000).

The most detailed analysis of GBS βH/C in the pathogenesis of septicemia was accomplished using WT GBS and isogenic mutants in the adult rabbit model (Ring *et al*., 2002a). Mortality was highest in rabbits challenged with an HH mutant (67%), compared to animals infected with the weakly hemolytic parent strain (27%) or the NH mutant (0%). Corresponding changes in bacterial counts in the blood and mean arterial pressure pressure confirmed the association of βH/C expression with resistance to phagocytic clearance and induction of sepsis symptomatology. Histopathologically, disseminated septic microabscesses surrounded by necrotic foci were found exclusively in the livers of animals infected with the HH mutant, and they also exhibited a 20-fold increase in serum transaminase levels and evidence of hepatocyte necrosis and apoptosis. Together, the work provided clear evidence that GBS βH/C plays a crucial role in the pathophysiology of GBS sepsis by inducing liver failure and high mortality (Ring *et al*., 2002a).

Meningitis

A feared but not infrequent manifestation of invasive GBS disease is meningitis. Meningitis is a significant cause of mortality in GBS disease and 25–40% of infants may suffer permanent neurological sequelae, such as hearing loss, cognitive delay, seizures, or cerebral palsy. In animal models, the risk of meningitis can be correlated to the magnitude and duration of GBS bacteremia (Ferrieri *et al*., 1980), and the known effects of βH/C in blocking phagocytic clearance (Liu *et al*., 2004) would be predicted to increase the chance of meningitis on this account alone. GBS also have the ability to invade and transcytose human brain endothelial cells (Nizet *et al*., 1997b), the single cell layer that comprises the majority of the blood-brain barrier. The ability of the βH/C to stimulate GBS intracellular invasion (Doran *et al*., 2002) could be seen as a second potential factor promoting the development of meningitis. Finally, GBS has been shown to activate inflammatory gene expression in human brain endothelial cells and to promote the consequent recruitment, activation, and transcytosis of neutrophils (Doran *et al*., 2003). Inflammatory damage to the blood-brain barrier induced by the βH/C therefore represents a third mechanism by which the toxin could promote the development of meningitis.

Consistent with the above mechanisms identified by *in vitro* experiments, mice challenged *in vivo* with an isogenic ΔβH/C mutant experience less blood-brain barrier penetration and suffer fewer cases of meningitis than mice challenged with the WT GBS parent strain (Doran *et al*., 2003). Bacterial counts in brain tissue were significantly higher in the animals challenged with WT GBS, and histopathologic studies confirmed meningeal thickening, thrombosis of meningeal vessels, leukocytic infiltration, and areas of bacterial microabscess formation in the brain parenchyma, changes generally absent in mice challenged with the ΔβH/C mutant. Additional studies performed by intrathecal challenge of rats with GBS WT and ΔβH/C mutant strains have shown a direct correlation of toxin expression to apoptosis of hippocampal neurons (J. Weber and V. Nizet, unpublished), a point of clinical concern given the limited plasticity of neuronal cells for regeneration.

Potential therapeutics

Because the βH/C contributes directly to disease pathogenesis through direct cellular injury, resistance to immune clearance, and activation of local and systemic inflammatory responses, the toxin constitutes an attractive target for adjunctive therapy of infants with GBS infection. Neutralizing antibody to the βH/C does not arise in the course of natural infection (Todd, 1934), and no specific antisera were detected upon repeated injection of partially purified βH/C preparations in rabbits for three months (Dal and Monteil, 1983). Progress in development of antibodies may benefit from more detailed knowledge of protein structure, as a synthetic peptide approach has recently been successful in generating inhibitory antibodies against the naturally non-immunogenic SLS toxin of GAS (Carr *et al*., 2001; Dale *et al*., 2002).

An alternative therapeutic modality to GBS infection may involve the use of phospholipid inhibitors to inactivate the βH/C. DPPC has been shown in many *in vitro* studies to be highly effective in blocking lysis of erythrocytes, fibroblasts epithelial cells, and macrophages (Tapsall and Phillips, 1991; Nizet *et al*., 1996; Liu and Nizet, 2004). Likewise, cytokine secretion, apoptosis, and cellular invasion promoted by the GBS βH/C can also be prevented using this inhibitor

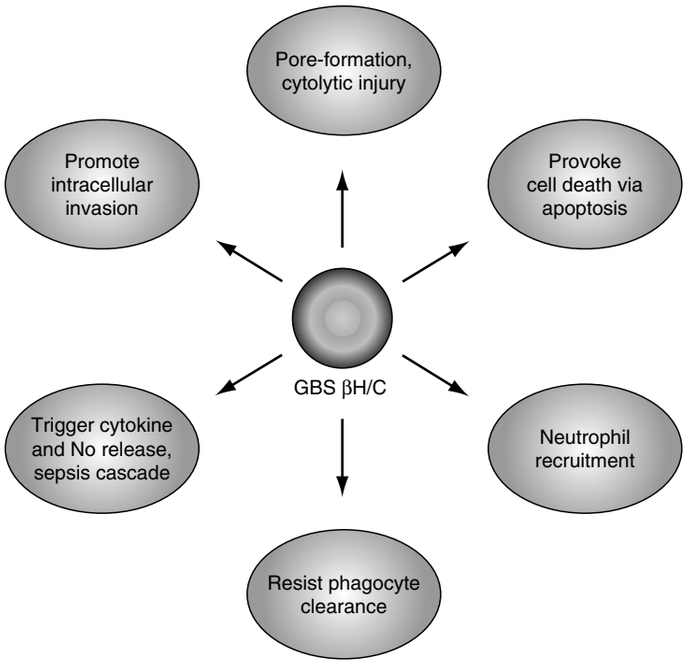

FIGURE 43.3 Schematic representation of biological activities attributed to the GBS β-hemolysin/cytolysin, a multifunctional toxin and virulence factor in the pathogenesis of invasive infection.

(Fettucciari *et al*., 2000; Doran *et al*., 2002; Liu *et al*., 2004). DPPC is a major component of surfactant, which is used therapeutically to restore lung compliance in premature neonates. Indeed, experiments in a ventilated premature rabbit model of GBS pneumonia and retrospective analysis of clinical data from human neonates with early onset GBS suggest beneficial effects of surfactant therapy against GBS-induced lung injury (Herting *et al*., 1997; Herting *et al*., 2000). Recent data identifying a key role of the βh/c in newborn pneumonia and lung injury (Hensler *et al*., 2005) may provide a key molecular mechanism explaining the therapeutic efficacy of surfactant replacement in terms of DPPC inhibition of the toxin.

CONCLUSION

Fueled by technological innovations in streptococcal genetics, the past decade has witnessed a resurgence of research into the biology and function of the GBS βH/C. The role of the βH/C as a virulence factor in disease pathogenesis has been confirmed in many animal models of GBS infection, and several unique properties of the toxin have been characterized. It is apparent that the βH/C is a multifunctional molecule, capable of exerting cytolytic, proapoptotic, proinvasive, proinflammatory, or antiphagocytic effects on a variety of target cells (Figure 43.3). At the biochemical level, select signaling pathways triggered by the βH/C are beginning to be unraveled. Yet the basic structural properties of the toxin remain poorly characterized because of difficulties presented in purification of this labile and apparently non-immunogenic toxin. Determination of structure of the mature protein toxin represents the overriding challenge for the future of GBS βH/C research.

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REFERENCES

Atwood, K.C. and Norman, A. (1949). On the interpretation of multihit survival curves. *Proc. Natl. Acad. Sci. USA* **35,** 696–702.

Berner, R. (2004). Significance, management, and prevention of *Streptococcus agalactiae* infection during the perinatal period. *Expert. Rev. Anti. Infect. Ther*. **2,** 427–437.

Bernheimer, A.W. (1947). Comparative kinetics of hemolysis induced by bacterial and other hemoysins. *J. Gen. Physiolol*. **30,** 337–353.

Blancas, D., Santin, M., Olmo, M., Alcaide, F., Carratala, J. and Gudiol, F. (2004). Group B streptococcal disease in non-pregnant adults: incidence, clinical characteristics, and outcome. *Eur. J. Clin. Microbiol. Infect. Dis*. **23,** 168–173.

Carr, A., Sledjeski, D.D., Podbielski, A., Boyle, M.D. and Kreikemeyer, B. (2001). Similarities between complement-mediated and streptolysin S-mediated hemolysis. *J. Biol. Chem*. **276,** 41790–41796.

Cornelis, G.R. and Van Gijsegem, F. (2000). Assembly and function of type III secretory systems. *Annu. Rev. Microbiol*. **54,** 735–774.

Dal, M.C. and Monteil, H. (1983). Hemolysin produced by group B *Streptococcus agalactiae. FEMS Microbiol. Lett*. **16,** 89–94.

Dale, J.B., Chiang, E.Y., Hasty, D.L. and Courtney, H.S. (2002). Antibodies against a synthetic peptide of SagA neutralize the cytolytic activity of streptolysin S from group A streptococci. *Infect. Immun*. **70,** 2166–2170.

Doran, K.S., Chang, J.C., Benoit, V.M., Eckmann, L. and Nizet, V. (2002). Group B streptococcal beta-hemolysin/cytolysin promotes invasion of human lung epithelial cells and the release of interleukin-8. *J. Infect. Dis*. **185,** 196–203.

Doran, K.S., Liu, G.Y. and Nizet, V. (2003). Group B streptococcal β-hemolysin/cytolysin activates neutrophil signaling pathways in brain endothelium and contributes to development of meningitis. *J. Clin. Invest*. **112,** 736–744.

Doran, K.S. and Nizet, V. (2004). Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy. *Mol. Microbiol*. **54,** 23–31.

Duncan, J.L. and Mason, L. (1976). Characteristics of streptolysin S hemolysis. *Infect. Immun*. **14,** 77–82.

Felmlee, T., Pellett, S. and Welch, R.A. (1985). Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. *J. Bacteriol*. **163,** 94–105.

Ferrieri, P. (1982). *Basic Concepts of Streptococci and Streptococcal Diseases*. Reedbooks: Surrey.

Ferrieri, P., Burke, B. and Nelson, J. (1980). Production of bacteremia and meningitis in infant rats with group B streptococcal serotypes. *Infect. Immun*. **27,** 1023–1032.

Fettucciari, K., Fetriconi, I., Bartoli, A., Rossi, R. and Marconi, P. (2003). Involvement of mitogen-activated protein kinases in group B *Streptococcus*-induced macrophage apoptosis. *Pharmacol. Res*. **47,** 355–362.

Fettucciari, K., Rosati, E., Scaringi, L., Cornacchione, P., Migliorati, G., Sabatini, R., Fetriconi, I., Rossi, R. and Marconi, P. (2000). Group B *Streptococcus* induces apoptosis in macrophages. *J. Immunol*. **165,** 3923–3933.

Gibson, R.L., Nizet, V. and Rubens, C.E. (1999). Group B streptococcal beta-hemolysin promotes injury of lung microvascular endothelial cells. *Pediatr. Res*. **45,** 626–634.

Gilmore, M.S., Segarra, R.A. and Booth, M.C. (1990). An HlyB-type function is required for expression of the *Enterococcus faecalis* hemolysin/bacteriocin. *Infect. Immun*. **58,** 3914–3923.

Ginsburg, I. (1970). Streptolysin S. In: *Microbial Toxins, Vol. 3. Bacterial Protein Toxins* (eds. T.C. Montiel, S. Kadis, and S.J. Ajl), pp. 97–176. Academic Press, New York.

Glaser, P., Rusniok, C., Buchrieser, C., Chevalier, F., Frangeul, L., Msadek, T., Zouine, M., Couve, E., Lalioui, L., Poyart, C., Trieu-Cuot, P. and Kunst, F. (2002). Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. *Mol. Microbiol*. **45,** 1499–1513.

Glaser, P., Sakamoto, H., Bellalou, J., Ullmann, A. and Danchin, A. (1988). Secretion of cyclolysin, the calmodulin-sensitive adenylate cyclase-hemolysin bifunctional protein of Bordetella pertussis. *Embo J*. **7,** 3997–4004.

Griffiths, B.B. and Rhee, H. (1992). Effects of hemolysins of groups A and B streptococci on cardiovascular system. *Microbios* **69,** 17–27.

Hensler, M.E., Liu, G.Y., Sobczak, S., Bernirshcke, K., Nizet, V. and Heldt, G.P. (2005). Virulence role of the group B streptococcal β-hemolysin/cytolysin in a neonatal rabbit model of early onset pulmonary infection. *J. Infect. Dis. in press*.

Herting, E., Gefeller, O., Land, M., van Sonderen, L., Harms, K. and Robertson, B. (2000). Surfactant treatment of neonates with respiratory failure and group B streptococcal infection. Members of the Collaborative European Multicenter Study Group. *Pediatrics* **106,** 957–964; discussion 1135.

Herting, E., Sun, B., Jarstrand, C., Curstedt, T. and Robertson, B. (1997). Surfactant improves lung function and mitigates bacterial growth in immature, ventilated rabbits with experimentally-induced neonatal group B streptococcal pneumonia. *Arch. Dis. Child Fetal Neonatal. Ed*. **76,** F3–8.

Inoue, K., Akiyama, Y., Kinoshita, T., Higashi, Y. and Amano, T. (1976). Evidence for a one-hit theory in the immune bactericidal reaction and demonstration of a multihit response for hemolysis by streptolysin O and *Clostridium perfringens* theta-toxin. *Infect. Immun*. **13,** 337–344.

Lamy, M.-C., Zouine, M., Fert, J., Vergassola, M., Couve, E., Pellegrini, E., Glaser, P., Kunst, F., Msadek, T., Trieu-Cuot, P. and Poyart, C. (2004). CovS/CovR of group B streptococcus: a two-component global regulatory system involved in virulence. *Mol Microbiol in press*.

Liu, G.Y., Doran, K.S., Lawrence, T., Turkson, N., Puliti, M., Tissi, L. and Nizet, V. (2004). Sword and shield: Linked group B streptococcal β-hemolysin/cytolysin and carotenoid pigment function to subvert host phagocyte defense. *Proc. Natl. Acad. Sci. USA* **101,** 14491–14496.

Liu, G.Y. and Nizet, V. (2004). Extracellular virulence factors of group B streptococci. *Front Biosci*. **9,** 1794–1802.

Madden, J.C., Ruiz, N. and Caparon, M. (2001). Cytolysin-mediated translocation (CMT): a functional equivalent of type III secretion in Gram-positive bacteria. *Cell* **104,** 143–152.

Malley, R., Henneke, P., Morse, S.C., Cieslewicz, M.J., Lipsitch, M., Thompson, C.M., Kurt-Jones, E., Paton, J.C., Wessels, M.R. and Golenbock, D.T. (2003). Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc. Natl. Acad. Sci. USA* **100,** 1966–1971.

Marchlewicz, B.A. and Duncan, J.L. (1980). Properties of a hemolysin produced by group B streptococci. *Infect. Immun*. **30,** 805–813.

Marchlewicz, B.A. and Duncan, J.L. (1981). Lysis of erythrocytes by a hemolysin produced by a group B *Streptococcus* sp. *Infect. Immun*. **34,** 787–794.

Merritt, K. and Jacobs, N.J. (1978). Characterization and incidence of pigment production by human clinical group B streptococci. *J. Clin. Microbiol*. **8,** 105–107.

Morbidoni, H.R., de Mendoza, D. and Cronan, J.E., Jr. (1996). *Bacillus subtilis* acyl carrier protein is encoded in a cluster of lipid biosynthesis genes. *J. Bacteriol*. **178,** 4794–4800.

Nizet, V. (2002). Streptococcal β-hemolysins: genetics and role in disease pathogenesis. *Trends Microbiol*. **10,** 575–580.

Nizet, V., Gibson, R.L., Chi, E.Y., Framson, P.E., Hulse, M. and Rubens, C.E. (1996). Group B streptococcal beta-hemolysin expression is associated with injury of lung epithelial cells. *Infect. Immun*. **64,** 3818–3826.

Nizet, V., Gibson, R.L. and Rubens, C.E. (1997a). The role of group B streptococci β-hemolysin expression in newborn lung injury. *Adv. Exp. Med. Biol*. **418,** 627–630.

Nizet, V., Kim, K.S., Stins, M., Jonas, M., Chi, E.Y., Nguyen, D. and Rubens, C.E. (1997b). Invasion of brain microvascular endothelial cells by group B streptococci. *Infect. Immun*. **65,** 5074–5081.

Nizet, V. and Rubens, C.E. (2000). Pathogenic mechanisms and virulence factors of group B streptococci. In: *The Gram-Positive Pathogens* (ed. R.N. VA Fischetti, JJ Ferretti, DA Portnoy, JI Rood), pp. 125–136. ASM Press, Washington, D.C.

Platt, M.W. (1995). *In vivo* hemolytic activity of group B *Streptococcus* is dependent on erythrocyte-bacteria contact and independent of a carrier molecule. *Curr. Microbiol*. **31,** 5–9.

Pritzlaff, C.A., Chang, J.C., Kuo, S.P., Tamura, G.S., Rubens, C.E. and Nizet, V. (2001). Genetic basis for the β-hemolytic/cytolytic activity of group B *Streptococcus. Mol. Microbiol*. **39,** 236–247.

Puliti, M., Nizet, V., von Hunolstein, C., Bistoni, F., Mosci, P., Orefici, G. and Tissi, L. (2000). Severity of group B streptococcal arthritis is correlated with beta-hemolysin expression. *J. Infect. Dis*. **182,** 824–832.

Rawlings, M. and Cronan, J.E., Jr. (1992). The gene encoding *Escherichia coli* acyl carrier protein lies within a cluster of fatty acid biosynthetic genes. *J. Biol. Chem*. **267,** 5751–5754.

Ring, A., Braun, J.S., Nizet, V., Stremmel, W. and Shenep, J.L. (2000). Group B streptococcal beta-hemolysin induces nitric oxide production in murine macrophages. *J. Infect. Dis*. **182,** 150–157.

Ring, A., Braun, J.S., Pohl, J., Nizet, V., Stremmel, W. and Shenep, J.L. (2002a). Group B streptococcal beta-hemolysin induces mortality and liver injury in experimental sepsis. *J. Infect. Dis*. **185,** 1745–1753.

Ring, A., Depnering, C., Pohl, J., Nizet, V., Shenep, J.L. and Stremmel, W. (2002b). Synergistic action of nitric oxide release from murine macrophages caused by group B streptococcal cell wall and beta-hemolysin/cytolysin. *J. Infect. Dis*. **186,** 1518–1521.

Ross, R.A., Madoff, L.C. and Paoletti, L.C. (1999). Regulation of cell component production by growth rate in the group B *Streptococcus. J. Bacteriol*. **181,** 5389–5394.

Shen, Z. and Byers, D.M. (1996). Isolation of *Vibrio harveyi* acyl carrier protein and the *fab*G, *acp*P, and *fab*F genes involved in fatty acid biosynthesis. *J. Bacteriol*. **178,** 571–573.

Spellerberg, B., Martin, S., Brandt, C. and Lutticken, R. (2000). The *cyl* genes of *Streptococcus agalactiae* are involved in the production of pigment. *FEMS Microbiol. Lett*. **188,** 125–128.

Spellerberg, B., Pohl, B., Haase, G., Martin, S., Weber-Heynemann, J. and Lutticken, R. (1999). Identification of genetic determinants for the hemolytic activity of *Streptococcus agalactiae* by ISS1 transposition. *J. Bacteriol*. **181,** 3212–3219.

Tapsall, J.W. (1986). Pigment production by Lancefield group B streptococci (*Streptococcus agalactiae*). *J. Med. Microbiol*. **21,** 75–81.

Tapsall, J.W. (1987). Relationship between pigment production and hemolysin formation by Lancefield group B streptococci. *J. Med. Microbiol*. **24,** 83–87.

Tapsall, J.W. and Phillips, E.A. (1991). The hemolytic and cytolytic activity of group B streptococcal hemolysin and its possible role in early onset group B streptococcal disease. *Pathology* **23,** 139–144.

Tettelin, H., Masignani, V., Cieslewicz, M.J., Eisen, J.A., Peterson, S., Wessels, M.R., Paulsen, I.T., Nelson, K.E., Margarit, I., Read, T.D., Madoff, L.C., Wolf, A.M., Beanan, M.J., Brinkac, L.M., Daugherty, S.C., DeBoy, R.T., Durkin, A.S., Kolonay, J.F., Madupu, R., Lewis, M.R., Radune, D., Fedorova, N.B., Scanlan, D., Khouri, H., Mulligan, S., Carty, H.A., Cline, R.T., Van Aken, S.E., Gill, J., Scarselli, M., Mora, M., Iacobini, E.T., Brettoni, C., Galli, G., Mariani, M., Vegni, F., Maione, D., Rinaudo, D., Rappuoli, R., Telford, J.L., Kasper, D.L., Grandi, G. and Fraser, C.M. (2002). Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V *Streptococcus agalactiae. Proc. Natl. Acad. Sci. USA* **99,** 12391–12396.

Todd, E.W. (1934). A comparative serological study of streptolysins derived from human and animal infections with note on pneumococcal hemolysin, tetanolysin, and streptococcal toxin. *J. Pathol. Bacteriol*. **39,** 299–321.

Tsaihong, J. and Wennerstrom, D. (1983). Effect of carrier molecules on production and properties of extracellular hemolysin produced by *Streptococcus agalactiae. Curr. Microbiol*. **9,** 333–338.

Ulett, G.C., Bohnsack, J.F., Armstrong, J. and Adderson, E.E. (2003). Beta-hemolysin-independent induction of apoptosis of macrophages infected with serotype III group B *Streptococcus. J. Infect. Dis*. **188,** 1049–1053.

Weiser, J.N. and Rubens, C.E. (1987). Transposon mutagenesis of group B streptococcus beta-hemolysin biosynthesis. *Infect. Immun*. **55,** 2314–2316.

Wennerstrom, D.E., Tsaihong, J.C. and Crawford, J.T. (1985). Evaluation of the role of hemolysin and pigment in the pathogenesis of early onset group B streptococcal infection. In: *Recent Advances in Streptococci and Streptococcal Diseases* (eds. Y. Kimura, S. Kotami, and Y. Shiokowa, Bracknell), pp. 155–156. Reedbooks, U.K.