β-Hemolysin–Independent Induction of Apoptosis of Macrophages Infected with Serotype III Group B Streptococcus

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Group B streptococcus (GBS) induces apoptosis in macrophages. Growth conditions minimizing β-hemolysin expression, such as high glucose, reduce apoptosis. We constructed an isogenic mutant strain of GBS 874391 lacking the β-hemolysin structural gene *cylE* and investigated the role that β-hemolysin plays in apoptosis of J774 macrophages. Viability of macrophages infected with wild-type or *cylE* GBS was similar and significantly less than that of macrophages infected with GBS grown in high-glucose media. Thus, apoptosis in GBS-infected macrophages is dependent not on β-hemolysin but on a factor coregulated with β-hemolysin by glucose.

Streptococcus agalactiae (group B streptococcus [GBS]) persists as a common cause of serious bacterial infections in newborns [1]. Neonates acquire GBS from colonized mothers by aspiration of infected amniotic fluid or vaginal secretions at birth [1]. Poor neutrophil chemotaxis and low levels of complement and specific antibodies directed against the capsular polysaccharide of GBS may predispose newborns to disease [1]. The increased susceptibility of neonates to GBS infection, compared with that of adults, may also be related to deficiencies in alveolar macrophage bactericidal function [2, 3]. Serotype III GBS can persist inside macrophages for extended periods after nonopsonic phagocytosis [4, 5]. Persistence of serotype III GBS within macrophages and manipulation of host cell antimicrobial activity may contribute to defective inflammatory responses in the lung during infection. Moreover, because therapy of GBS infection in neonates generally involves antibiotics with little intraphagocytic penetration, persistence within an intracellular niche could allow GBS to evade antibiotic killing [6].

Fettucciari et al. [7] demonstrated that serotype III GBS induces apoptosis in infected macrophages. Their investigation showed that down-regulation of β-hemolysin expression, by growth of GBS in glucose-supplemented media, inhibited macrophage apoptosis, which suggests that the surface-bound β-hemolysin of GBS is the bacterial factor responsible for stimulating apoptosis in infected macrophages [7]. However, there has been no direct demonstration that GBS β-hemolysin plays a role in apoptosis of macrophages. In the present study, we investigated the role that β-hemolysin plays in nonopsonic phagocytosis, intracellular survival, and apoptosis in infected macrophages. We compared the effect of phagocytosis of a β-hemolysin–deficient isogenic mutant of serotype III GBS with that of the wild-type β-hemolytic parental strain and the ability of these strains to survive in macrophages and induce host-cell apoptosis. Growth conditions in which glucose levels were high were used to inhibit β-hemolysin expression in GBS strains, and the effect on the induction of macrophage cell death was assessed. The results demonstrate that apoptosis in serotype III GBS–infected macrophages does not depend on β-hemolysin per se but on a factor coregulated with β-hemolysin by glucose.

Materials and methods. GBS strains used in this study are described elsewhere [8] and were grown on Columbia blood agar (CBA) or Todd-Hewitt agar (THA) and in Todd-Hewitt broth (THB), with 10 μg/mL erythromycin for antibiotic selection, as indicated. The β-hemolysin–deficient isogenic mutant strain cyle9 was derived from strain 874391 by insertional deletion of the β-hemolysin structural gene, *cylE*. A 1109-bp fragment corresponding to sequence 5′ of the *cylE* coding sequence (cds; position 149, codon 50 of the *cylB* gene to position 383, codon 128 of *cylE*) and a 1055-bp fragment corresponding to the 3′ *cylE* terminus and flanking regions (position 1704, codon 568 of *cylE* to position 760, codon 253 of the *cylF* gene) were amplified by polymerase chain reaction by use of the following primer pairs: CYLPF2, 5′-GGA GTA CTG *(ata)* CAG TAT CCA GTG-3′ (position 149, codon 50 of the *cylB* gene), and CYLR1R2, 5′-TTG TAG TTA CTG AAT *(att)* TCG GTA TAG-3′ (position 360, codon 120 of *cylE*); and CYLR1F2, 5′-TCC AAG AGA CAG TGA ATT CTA *(ata)* TTC-3′ (position 1704, codon 568 of *cylE*), and CYLPR2, 5′-CAC TGG TGT

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TCC TGC *(tga)* AGC GAG-3′ (position 739, codon 246 of the *cylF* gene), respectively [9]. Underlined bases not matching the *cyl* gene cluster cds were incorporated into primers (from the original cds shown in parentheses) to produce specific restriction-endonuclease sites required for ligation. Amplification reactions were performed in 75-μL volumes containing 500 ng of genomic DNA, 0.2 mmol/L dNTPs, 3 mmol/L $MgCl_2$, 50 pmol of each primer, and 4 U of DNA polymerase (Bio-X-ACT; ISC BioExpress) in the manufacturer's supplied buffer. Thirty-five cycles, consisting of denaturation for 1 min at 94°C, annealing for 1.5 min at 50°C, and extension for 2.5 min at 72°C, were performed. Amplification products were digested with *EcoR1*, ligated, and reamplified with CYLPF2 and CYLPR2 primers, to yield an in-frame deletion of the *cylE* gene from bp 370–1716 inclusive. The mutant *cylE*$^-$ gene was cloned into the targeting vector pHY304 (provided by Dr. C. Rubens, University of Washington) and transformed into competent GBS strain 874391, which was grown at 30°C [10]. An erythromycin-resistant colony was grown in THB with erythromycin for 3 h at 30°C, and then bacteria were diluted 1:100 in fresh media, incubated for 3 h at 37°C, and plated onto THA containing erythromycin at 37°C. An erythromycin-resistant colony containing an integrated copy of the targeting vector was inoculated in 5 mL of THB without erythromycin, grown for 3 h at the permissive temperature of 30°C, plated on CBA, and incubated at 37°C. Genomic DNA was prepared from erythromycin-susceptible, nonhemolytic strain cyle9, and Southern blotting with a *cylE* probe was performed to confirm the deletion of the wild-type gene [11].

The murine macrophage–like cell line J774 was grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 0.5 μmol/L 2-mercaptoethanol (TCM), 100 U/mL penicillin, and 100 μg/mL streptomycin. Macrophages were seeded into 24-well cell-culture plates, at a concentration of 2.5×10^5 cells/well, and were incubated for 24 h at 37°C in 5% CO_2. Monolayers were washed twice with PBS and inoculated with GBS grown overnight in THB (washed 3 times in PBS and resuspended at a concentration of $2.5–3.5 \times 10^8$ cfu/mL in TCM). Preliminary experiments were performed using various MOI values, ranging from 1:1 (bacteria:macrophage) to 1000:1, to determine the optimal MOI of 100:1. Cultures were centrifuged at 500 *g* for 10 min, to enhance the association between macrophages and GBS. After a 2-h incubation period (37°C in 5% CO_2), challenge inocula were removed. Infected monolayers were washed twice with PBS; fresh TCM with penicillin, streptomycin, and gentamicin (100 μg/mL) was added; and macrophages were incubated for 0, 4, 24, 48, 72, or 96 h at 37°C in 5% CO_2. For colony counts of intracellular bacteria, monolayers ($n = 3$) were washed twice with PBS, and 0.01% Triton X in distilled water was used to lyse macrophages, which were diluted in PBS and dispensed onto THA. Data are expressed as the mean ± SEM colony-forming units per milliliter for 6 independent experiments. The number of GBS attached to the macrophage surface after the 2-h infection period was ~3 times greater than the number of GBS internalized (data not shown). Duplicate wells were used to assess macrophage viability by trypan blue exclusion ($n = 2$), and additional wells were used for apoptosis assays.

Hemolytic activity was determined by a modified sheep red blood cell (SRBC) assay [7]. In brief, bacteria were grown in THB overnight, washed 3 times in PBS, and resuspended at a concentration of $1.5–2.5 \times 10^9$ cfu/mL in PBS. Fifty microliters of bacteria was added to 5 mL of 1% SRBCs in THB and incubated for 3.5 h at 37°C. SRBCs incubated with THB plus PBS or THB plus 0.1% SDS were used as negative and positive controls, respectively. Cell suspensions were clarified (3000 *g* for 10 min), and optical density at 405 nm of supernatants was measured. To inhibit β-hemolytic activity of serotype III GBS, the bacteria were grown in THB supplemented with glucose (10 mg/mL) overnight [7, 12]. Preliminary experiments using various concentrations of glucose demonstrated that 10 mg/mL was the optimal concentration for inhibition of GBS β-hemolytic activity.

Apoptosis in macrophages was detected by 3 different methods: transmission electron microscopy to assess morphological changes, TUNEL staining (TdT-FragEL, QIA33), and DNA fragmentation assay (Suicide-Track, AM41). All reagents and kits used for the detection of apoptosis were purchased from Oncogene. For TUNEL staining, monolayers were cultured on 15-mm round glass coverslips (Thermanox), which were inserted into 24-well culture plates before culture. At the specified time points, monolayers were fixed in 4% formaldehyde for 10 min, dried in 80% ethanol, and frozen at −20°C until staining, which was performed in-well. After staining, coverslips were removed from wells, inverted, and mounted on glass slides in Consul-Mount (Shandon). The percentage of TUNEL-positive macrophages in each sample was determined by counting 4 40× fields under light microscopy (~200–400 cells/field).

Differences in the numbers of intracellular bacteria between GBS strains were compared using Student's *t* test (SPSS version 9.0; SPSS). Levels of macrophage mortality were compared using the nonparametric Kruskal-Wallis test (SPSS version 9.0). $P < .05$ was considered to be significant.

Results. Phagocytosis and intracellular survival of GBS 874391 and cyle9, in J774 macrophages, is shown in figure 1*A*. After the 2-h infection period, 6.7 ± 0.1 $\log_{10}$ cfu/mL of GBS 874391 and cyle9 were recovered from macrophages, indicating similar levels of phagocytosis of both GBS strains. Viable GBS of both strains persisted inside macrophages for 72 h. Macrophage mortality after infection, as assessed by trypan blue staining, is shown in figure 1*B*. During the first 24 h of culture, no significant differences in macrophage viability were observed

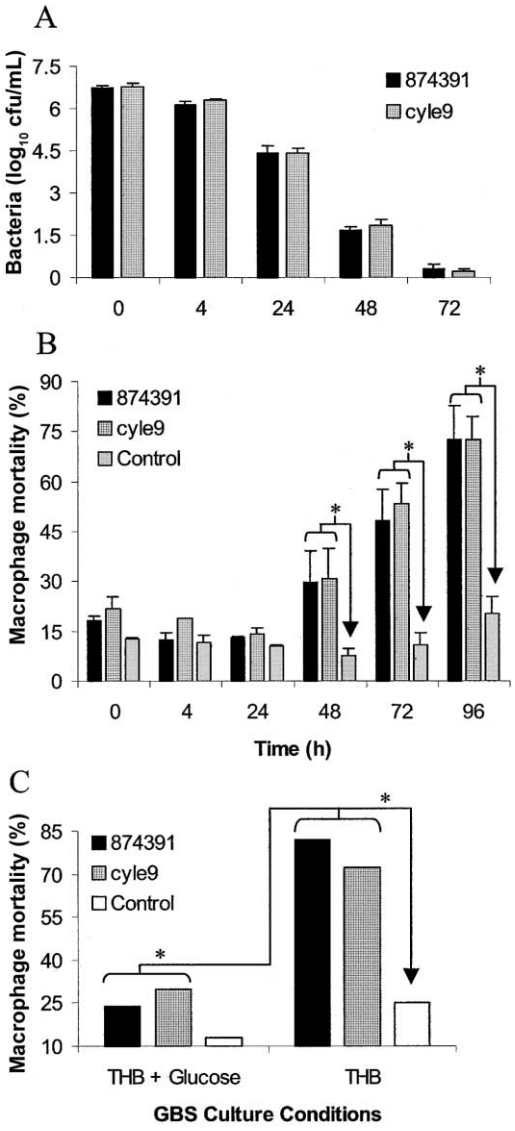

Figure 1. *A,* Phagocytosis and intracellular survival of serotype III group B streptococcus (GBS) 874391 and β-hemolysin–deficient isogenic mutant cyle9, in J774 macrophages. Macrophages (2.5×10^5 cells/well) were infected at an MOI of 100:1 (bacteria:macrophage); after a 2-h incubation period, extracellular bacteria were killed, and nos. of intracellular bacteria were determined by colony counts of cell lysates on Todd-Hewitt agar ($n = 3$). Data are mean ± SEM of 6 independent experiments. Equal nos. of 874391 and cyle9 were phagocytosed after the 2-h infection period, and both GBS strains persisted within macrophages in a viable form for 72 h. *B,* Cytotoxicity in infected and control macrophages was demonstrated by trypan blue staining. Levels of macrophage mortality increased at 48 h for GBS-infected macrophages, but there were no significant differences between the levels of mortality induced by the different strains of GBS at any time point. Significant differences in mortality between macrophages infected with either GBS strain and noninfected macrophages were present at 48, 72, and 96 h after infection. *C,* Infection of macrophages with GBS grown in Todd-Hewitt broth (THB) supplemented with 10 mg/mL glucose resulted in significantly less cytotoxicity at 96 h after infection, compared with macrophages infected with GBS grown in THB alone ($n = 3$). *$P < .05$.

between infected cells and noninfected controls. At 48 h and thereafter, macrophage mortality in infected cultures increased rapidly, reaching 73% ± 10% and 73% ± 7% for GBS 874391 and cyle9, respectively, at 96 h. Culture of GBS 874391 in glucose-supplemented THB overnight completely inhibited normal β-hemolytic activity of this GBS strain. The β-hemolytic activities of GBS strains were as follows: GBS 874391 grown in THB, $OD_{405} = 0.156$; GBS 874391 grown in THB supplemented with glucose, $OD_{405} = 0.000$; and cyle9 grown in THB with or without glucose, $OD_{405} = 0.000$. THB alone was not β-hemolytic ($OD_{405} = 0.000$), whereas 0.1% SDS resulted in an OD_{405} of 0.173. Mortality in macrophages infected with either GBS 874391 or cyle9 grown in glucose-supplemented THB was significantly lower, compared with mortality in macrophages infected with these GBS strains grown in THB alone (figure 1*C*). These data suggest that a glucose-regulated factor other than β-hemolysin is responsible for the induction of macrophage cell death.

Cellular morphology of macrophages infected with GBS 874391 and cyle9 is shown in figure 2*A*. Typical features of apoptotic cells are shown in macrophages infected with each GBS strain, including diploid nuclei, nuclear condensation, and membrane blebbing, but are absent in noninfected macrophages. Results of TUNEL staining of macrophages infected with GBS 874391 and cyle9 are shown in figure 2*B*. At 48 h after infection, the percentages of TUNEL-positive macrophages were 31% ± 3% and 25% ± 9% for GBS 874391 and cyle9, respectively. The percentage of TUNEL-positive macrophages in noninfected cultures was <3%. At 72 h after infection, extensive TUNEL-positive staining was observed in the majority of macrophages (data not shown). Results from DNA fragmentation assays are shown in figure 2*C*. DNA fragmentation was detected in macrophages infected with GBS strain 874391 and cyle9, beginning 48 h after infection and continuing for the duration of the experiment. DNA fragmentation was observed in positive control apoptotic macrophages (figure 2*C, lane S;* 20 μmol/L staurosporine for 3 h) but not in either untreated control macrophages (figure 2*C, lane U*) or necrotic macrophages (figure 2*C, lane N;* 0.2% saponin).

Discussion. An increasing number of bacterial pathogens have been identified as mediators of apoptosis [13], and, depending on the nature of the pathogen, this may be beneficial or detrimental to the host during pulmonary infection [14]. On the one hand, intracellular pathogens, such as *Chlamydia* and *Leishmania* species, may gain benefit by suppressing host-cell apoptosis to aid intracellular survival. On the other hand, extracellular pathogens, such as GBS, may enhance their chance of survival by inducing apoptosis of host cells to eliminate phagocytes, such as macrophages, and to subvert inflammation and the immune response [14]. Moreover, prolonged survival of GBS within macrophages may allow the bacteria to escape

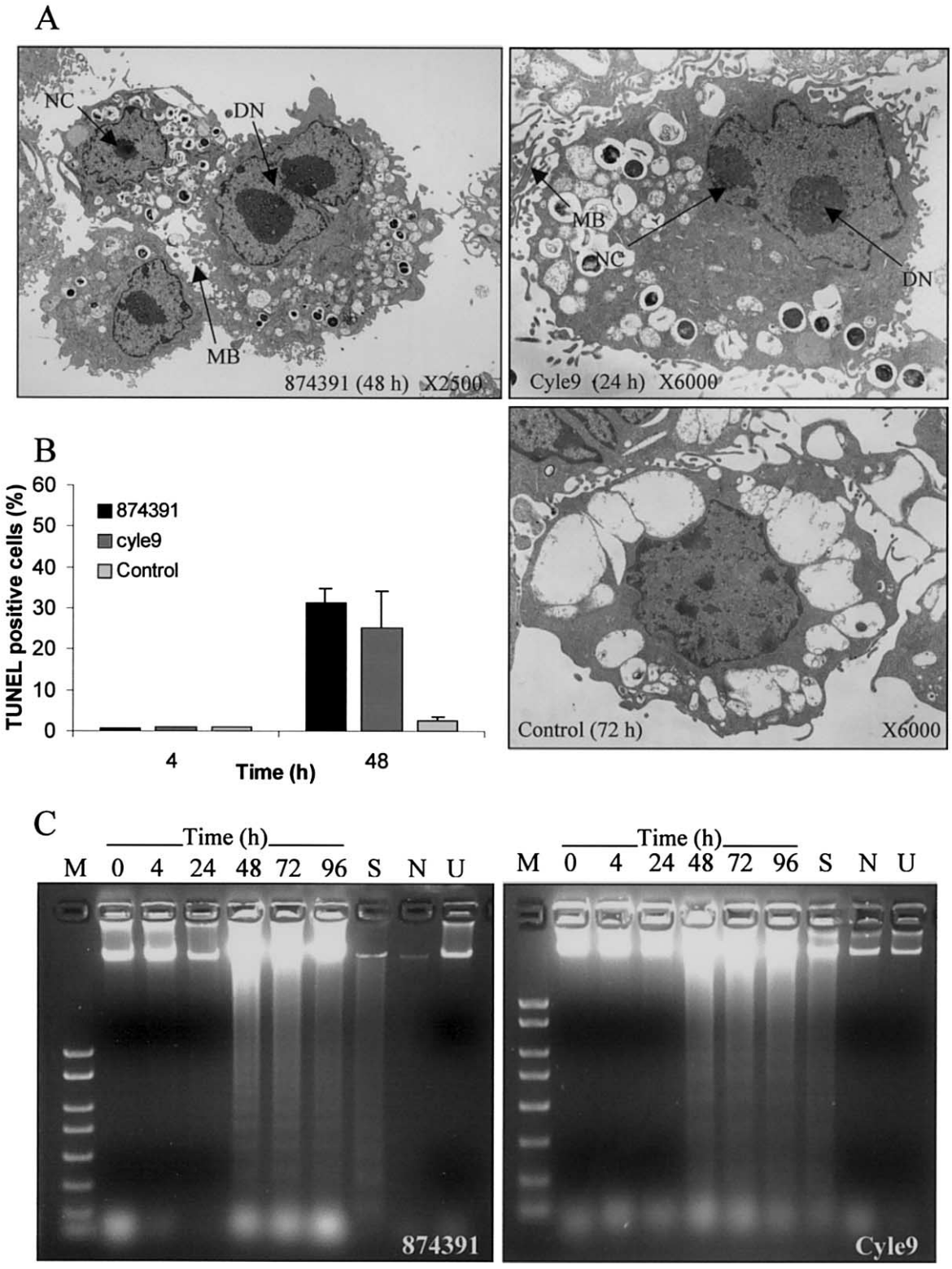

Figure 2. Apoptosis in J774 macrophages infected with either hemolytic group B streptococcus (GBS) 874391 or the nonhemolytic isogenic mutant cyle9. *A,* Morphology of macrophages showing characteristic features of apoptosis, including nuclear condensation (NC), diploid nuclei (DN), and membrane blebbing (MB) in cells after infection with either GBS strain at 24–48 h after infection. *B,* Equivalent percentages of TUNEL-positive macrophages at 48 h after infection ($n = 4$; mean $\pm$ SEM, no significant differences shown between GBS 874391 and the nonhemolytic isogenic mutant cyle9, as assessed using non-parametric Kruskal-Wallis test [SPSS version 9.0]). *C,* DNA fragmentation demonstrated in macrophages infected with either GBS 874391 or cyle9 ($n = 3$). *Lane M,* 100-bp DNA marker; *lane S,* staurosporine-treated macrophages (20 μmol/L for 3 h); *lane N,* saponin-treated macrophages (0.2% for 3 h); and *lane U,* untreated control macrophages.

phagocytosis by polymorphonuclear leukocytes and to avoid extracellular antibiotics and components of humoral immunity.

Fettucciari et al. [7] demonstrated GBS-induced apoptosis in macrophages after nonopsonic phagocytosis and showed that apoptosis was inhibited by growing the bacteria in conditions minimizing β-hemolysin expression. These observations suggested that apoptosis was attributable to GBS β-hemolysin [7]. The results from the present study, however, demonstrate that apoptosis in macrophages infected with serotype III GBS does not require β-hemolysin. When we inhibited β-hemolytic activity of wild-type GBS by culturing the bacteria in glucose-supplemented media [12], the level of cell death in infected macrophages was reduced significantly. This result is consistent with the previous report of GBS-induced apoptosis [7], which correlated glucose inhibition of GBS β-hemolytic activity with reduced apoptosis in infected macrophages. However, we constructed a β-hemolysin–deficient isogenic mutant of serotype III GBS and demonstrated equivalent levels of apoptosis in macrophages infected with this mutant, compared with those in the β-hemolytic wild-type parent strain. Furthermore, parallel experiments performed in the present study demonstrated that macrophage mortality in response to infection with the nonhemolytic isogenic mutant strain cyle9 also was inhibited to a similar degree after culture of the β-hemolysin–deficient mutant in high concentrations of glucose. The genetic approach we have used in the present study, in conjunction with experiments to inhibit β-hemolytic activity in culture, shows that serotype III GBS–induced apoptosis in macrophages is induced by a factor that is coregulated with β-hemolysin by glucose, rather than by β-hemolysin alone. Proapoptotic activity of serotype III GBS toward macrophages is associated with persistence of viable intracellular bacteria in macrophages for 72 h, which is consistent with results of other studies [4, 5]. In summary, the present study has shown that nonopsonic phagocytosis of serotype III GBS by macrophages results in apoptosis of the host cell, but, in contrast to a previous report, we have shown that this apoptosis is induced independently of the presence of β-hemolysin and is mediated by a factor coregulated with β-hemolysin by glucose.

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