Preterm birth is a major factor contributing to neonatal disease and accounts for 75% of perinatal mortality worldwide (Goldenberg et al., 2000). Currently, there is no effective therapy for prevention of human preterm births or stillbirths. Intra-amniotic infection and inflammation are important causes of preterm birth, stillbirth, fetal injury, and early onset neonatal sepsis (Watts et al., 1992; Goldenberg et al., 2000; Yoon et al., 2000; Lukacs et al., 2004; Behrman and Butler, 2007). Early onset sepsis in human newborns manifests within the first few hours of life, is fulminant, and is due to organisms acquired in utero with the amniotic fluid and neonatal blood infected with organisms commonly colonizing the lower genital tract such as Group B Streptococcus (GBS; Hillier et al., 1988; Romero et al., 1989c; Hillier et al., 1991; Puopolo, 2008; Gravett et al., 2010; Verani et al., 2010).

GBS are β-hemolytic, gram-positive bacteria that are a frequent cause of human newborn infections. Morbidities due to GBS infections include delayed development, vision and hearing loss, chronic lung disease, mental retardation, and cerebral palsy (Ledergerber, 2008). Despite the success of intrapartum antibiotic prophylaxis to prevent GBS transmission to the neonate during labor and delivery, in utero infections that occur earlier in pregnancy leading to stillbirth and preterm birth are not targeted by this approach and the burden of early onset sepsis in newborn infants remains substantial (Verani et al., 2010; Weston et al., 2011). Additional preventive therapies are urgently needed before the widespread use of antibiotics in pregnant women creates sufficient resistance such that our current antibiotics become ineffective.
A key factor limiting preventive strategies is insufficient knowledge of virulence mechanisms that promote infection of the amniotic cavity. The human placenta is a critical multicellular organ that protects the growing fetus from organisms colonizing the lower genital tract. As the placenta is the most species-specific mammalian organ (Ala-Kokko et al., 2000; Hutson et al., 2011), no animal placenta recapitulates the exact mechanistic and physical barriers of the human placenta. The widely accepted route of pathogen entry into the human amniotic fluid requires bacterial ascension through the cervix and breach of several placental layers including the decidua, chorion, amnion, and amniotic epithelium (Bourne, 1960; Goldenberg et al., 2000). Although invasion of the amniotic epithelium is critical for pathogen entry into the amniotic cavity, previous studies have indicated that GBS do not invade human amniotic epithelial cells (hAECs) which constitute the amniotic epithelium (Winram et al., 1998). Consequently, mechanisms and virulence factors that mediate ascending GBS infection from the lower genital tract into the amniotic cavity and fetus are not understood.

RESULTS

Hemolysin promotes GBS invasion of hAECs

As GBS has been isolated from amniotic fluid of women with intact chorioamniotic membranes (Bobitt and Ledger, 1977; Naeye and Peters, 1978; Winram et al., 1998; Goldenberg et al., 2000), we investigated mechanisms that promote GBS invasion and breach of amniotic epithelium and chorionamnion. We hypothesized that intra-amniotic GBS infections in patients with intact placental or chorioamniotic membranes (Bobitt and Ledger, 1977; Naeye and Peters, 1978; Winram et al., 1998; Goldenberg et al., 2000) may be due to elevated virulence factor expression. The two component regulatory system CovR/S was described to repress the expression of many GBS virulence genes including genes of the cyl operon containing cylE important for the pluripotent toxin known as β-hemolysin/cytoleukin (hereafter referred to as hemolysin; Lamy et al., 2004; Jiang et al., 2005; Rajagopal et al., 2006). To test if increased expression of virulence factors promotes GBS invasion of amniotic epithelium, we compared the ability of WT GBS and the hyper-hemolytic ΔcovR to adhere to and invade hAEC. To evaluate the role of hemolysin, nonhemolytic GBS lacking the cylE gene associated with hemolysin production (Pritzlaff et al., 2001) were included (ΔcylE and ΔcovRΔcylE). The hemolytic activity of WT, hyper-hemolytic ΔcovR, and nonhemolytic ΔcylE strains is shown in Fig. 1 A. Primary hAECs were isolated and cultured from normal, term placentas obtained immediately after cesarean delivery from women without labor and adherence and invasion of GBS to hAEC was determined as previously described (Winram et al., 1998; Lembo et al., 2010). Consistent with previous reports (Winram et al., 1998), we observed that WT GBS adhered to hAEC (Fig. 1 B). The presence or absence of CovR or CylE had no significant effect on GBS adherence to hAEC (Fig. 1 B, P > 0.2). However, in contrast to previous observations (Winram et al., 1998), we observed that WT GBS invaded hAEC (~4% invasion; Fig. 1 C). The ΔcylE mutant showed significantly decreased invasion when compared with WT (~0.3% invasion, P = 0.008; Fig. 1 C). Consistent with our hypothesis, we observed that the hyper-hemolytic GBSΔcovR was significantly more invasive to hAEC when compared with the WT (~80% invasion, P < 0.0001; Fig. 1 C). Notably, the increase in hAEC invasion observed with GBSΔcovR was abolished in the absence of the gene cylE linked to hemolysin expression (Fig. 1 C, ΔcovRΔcylE). Collectively, these results indicate that hemolysin promotes GBS invasion of hAECs. Of note, the levels of GBS invasion observed with hAEC and differences between
hypoimvasive and hyperinvasive strains is consistent with levels of GBS invasion reported in other cell types including human brain microvascular endothelial cells and lung epithelial cells (Doran et al., 2002, 2005; van Sorge et al., 2009).

**Hemolysin induces activation of proinflammatory mediators in human amniotic epithelium**

We examined if increased hemolysin in ΔcovR activates an inflammatory response in human amniotic epithelium. To evaluate changes in expression of inflammatory genes in GBS-infected hAEC, qRT-PCR was performed on RNA isolated at 4 h after infection using previously described methods (Lembo et al., 2010). These results indicate that infection with ΔcovR caused a significant increase in transcription of cytokines such as IL-6, IL-8, IL-1β, CXCL1, and CCL20 in hAEC compared with cells infected with the isogenic WT (COH1) or uninfected controls (Fig. 2 A). Interestingly, the increase in inflammatory gene expression observed with GBSΔcovR was abolished in hAEC infected with ΔcovRΔcylE (Fig. 2 A). Luminex bead assays confirmed that secretion...
of IL-6, IL-8, and IL-1β was higher in ΔcovR-infected hAEC compared with the WT GBS or ΔcovRΔcylE (Fig. 2B, P < 0.005).

**Hyper-hemolytic GBSΔcovR infection increases NF-κB recruitment into the nucleus of hAEC**

Microbial toxins have been implicated in activating inflammatory signaling pathways via the nuclear transcription factor NF-κB, which is recruited from the cytoplasm to the nucleus during activation (Gonzalez et al., 2008). Therefore, we examined whether the increase in proinflammatory gene expression observed in hAEC infected with the hyper-hemolytic GBSΔcovR was associated with nuclear localization/recruitment of the transcription factor NF-κB. To test this hypothesis, total nuclear and cytoplasmic proteins isolated from infected and uninfected hAEC were resolved on 10% SDS-PAGE and Western blots were performed using antibody to NF-κB p65. The results shown in Fig. 2C indicate that infection with ΔcovR results in an ∼2.5-fold increase in recruitment of NF-κB into the nucleus of infected hAEC when compared with WT GBS or uninfected controls. These data confirm that the increase in inflammatory gene expression observed in ΔcovR-infected hAEC is associated with increased nuclear recruitment of NF-κB.

**Hemolysin promotes GBS breach of the human amniotic epithelial barrier**

We next examined if hemolysin accelerates failure of the human amniotic epithelial barrier. Changes in transepithelial electrical resistance were monitored across hAEC monolayers in real time using electric cell-substrate impedance sensing (ECIS; Gaeaver and Keese, 1993). In brief, hAEC monolayers established on gold-plated electrodes in 8-well array slides were infected with GBS WT, isogenic ΔcovR, ΔcovRΔcylE, or ΔcylE at 10⁵ CFU/well as previously described (Lembo et al., 2010). Uninfected wells were included as controls. Fig. 2D shows that the decrease in barrier resistance observed in hAEC infected with WT GBS was not observed in hAEC infected with the hemolysin-deficient strain ΔcylE. Furthermore, we observed that infection with ΔcovR accelerated the decrease in barrier resistance compared with WT (Fig. 2D). Notably, the rapid decrease in barrier resistance due to ΔcovR was abolished in ΔcovRΔcylE (Fig. 2D). These results suggest that increased hemolysin expression enables GBS to breach barrier function of the amniotic epithelium. We further observed that prolonged exposure of ΔcovR to hAEC (>4 h) induced cytotoxic effects in contrast to hAEC exposed to GBS WT and ΔcylE strains. Collectively, these observations indicated that hemolysin is an important virulence factor that promotes bacterial invasion and immune activation of the amniotic epithelium leading to secretion of cytokines, such as IL-6, IL-8, and IL-1β, that have associated with preterm labor and neonatal morbidity (Romero et al., 1989a,b, 1990, 1991; Gotsch et al., 2007).

**Hyper-hemolytic GBS penetrate human placenta/chorioamnion and can be associated with women in preterm labor**

Ascending in utero infection of GBS from the lower genital tract into the amniotic cavity requires the pathogen to penetrate the chorioamniotic membranes. Thus, we tested the ability of GBS to penetrate intact membranes that were mounted and maintained on a modified transwell system as previously described (Zaga-Clavellina et al., 2007). 48 h after stabilization, intact chorioamniotic membranes (n = 6) were infected with 10⁷ CFU of GBS (WT, ΔcovR, and ΔcovRΔcylE) on the choriodecidual side of the placenta. Uninfected chorioamnion was included as controls. In parallel, we also examined the ability of GBS to penetrate either chorion alone or amnion alone, using the transwell model system (for details, see Materials and methods). At 24 h after infection, aliquots of media from the lower chamber were analyzed for bacterial CFU. As shown in Fig. 3A, we observed that all GBS strains efficiently penetrated either the amnion or the chorion, as ≥10⁷ CFU was recovered from the lower chamber of membranes infected with WT, ΔcovR, and ΔcovRΔcylE. In contrast, no bacterial CFU was recovered from chorioamnion infected with WT or ΔcovRΔcylE (Fig. 3A). Notably, ≥10⁵ bacterial CFU were recovered from four of six chorioamniotic membranes infected with ΔcovR (Fig. 3A, P = 0.02). Transverse histological sections of the infected chorioamnion were prepared and stained for bacteria as previously described (Stevens and Bancroft, 1977). Interestingly, bacterial invasion of the chorioamnion, including penetration of the amniotic epithelium, was observed in chorioamniotic membranes infected with the hyper-hemolytic GBSΔcovR even when bacteria were not recovered in the lower chamber (Fig. 3B). In contrast, bacteria were primarily seen in the choriodecidual region in chorioamniotic membranes infected with WT and very few bacteria are observed in membranes infected with ΔcovRΔcylE (Fig. 3B). Increased secretion of IL-6 was also observed in media obtained from the lower chamber of chorioamniotic membranes infected with ΔcovR (Fig. 3C, P = 0.02). We were unable to extend the experiments longer than 24 h after GBS infection as the placental membranes begin to destabilize at >75 h after cesarean section. Collectively, these data show that although chorioamniotic membranes serve as an effective barrier to prevent GBS trafficking, increased production of hemolysin can facilitate bacterial penetration of chorioamniotic membranes and the amniotic cavity.

We examined if increased hemolytic activity could be observed in GBS isolated from women in preterm labor. To this end, clinical isolates obtained from human amniotic fluid and chorioamnion were examined for their hemolytic properties and potential mutations in the covR/S locus. We obtained eight GBS strains that were isolated from six women enrolled in a cohort of women in preterm labor with intact membranes and where information on outcomes and microbiological cultures of the amniotic fluid, chorioamnion, and cord blood were available (Hitti et al., 1997; see Table 1). Most of these GBS isolates exhibited increased hemolytic activity and increased transcription of genes in the cly operon such as clyE (Figs. 4, A and B). DNA sequencing indicated the presence of mutations in the covR/S loci in six isolates obtained from four women (Table 1). GBS isolated from both the amniotic fluid and chorioamnion of one patient in preterm labor
Of note, two GBS isolates recovered from women in preterm labor had no mutations in the **covR/S** loci, and thus increased hemolytic activity and transcription of **cyl** genes (Fig. 4, A and B) may be mediated by other regulators of GBS hemolysin.

To determine if the identified CovR/S mutations affect hemolytic activity of GBS, we generated site-directed mutants. As shown in Fig. 4 C, GBSΔcovR and ΔcovS strains had a stop codon mutation in the kinase domain of CovS (CovS220Stop; Table 1). GBS isolated from amniotic fluid and cord blood of another patient in preterm labor had a valine to methionine substitution in CovS at position 343; the same mutation was also observed in a GBS isolate obtained from the amniotic fluid of a third patient (Table 1). GBS from amniotic fluid of another woman in preterm labor had a deletion in the promoter of **covR/S** (Table 1).
exhibit increased hemolytic activity when compared with the WT and complementation with plasmids that constitutively express either CovR or CovS restored repression of hemolytic activity to WT levels or greater. However, complementation of ΔcovS with the plasmid encoding CovS220Stop failed to restore repression of hemolytic activity and partial complementation was observed with CovSV343M (compare ΔcovS/pCovS to ΔcovS/CovS220Stop and ΔcovS/CovSV343M in Fig. 4 C). Further studies are necessary to understand the role of this substitution in CovS signaling. Similarly, the presence of the promoter deletion in covR/S on the chromosome of WT GBS alleviated CovR repression of hemolysin (Fig. 4 C) and is consistent with our previous observations that CovR can positively regulate its own expression (Lembo et al., 2010). These results suggest that the increased hemolytic activity observed in the clinical isolates with CovR/S mutations can, at least in part, be attributed to these mutations. However, as GBS has an open pan-genome (Tettelin et al., 2005), the effect of other strains specific regulators cannot be ruled out. We further confirmed that, similar to ΔcovR, GBSΔcovS showed increased invasion and accelerated barrier disruption of hAEC (Figs. 4, D and E). Complementation of ΔcovR and ΔcovS decreased amniotic epithelial invasion at levels similar to WT (Fig. 4 D) as did the complemented ΔcylE strain (not depicted). Although we were unable to complement the ΔcovRΔcylE double mutant due to the instability of the complementing plasmid, introduction of the plasmid encoding CylE to GBSΔcovRΔcylE restored hemolytic activity, amniotic epithelial invasion, and barrier disruption to levels that were intermediate between WT and ΔcovR (Figs. 4, C–E).

CylE expression is necessary but not sufficient for GBS hemolysis

Our results above add to the large body of literature demonstrating the essential nature of hemolysin to various facets of GBS pathogenesis including pneumonia, sepsis, meningitis (Liu et al., 2004; Hensler et al., 2005; Lembo et al., 2010), and now bacterial penetration of human placenta/chorioamnion. Despite the importance of hemolysin to GBS virulence, its biochemical and molecular nature has remained elusive. Extraction of the GBS hemolysin requires high molecular weight stabilizers such as starch, tween, or BSA (Marchlewicz and Duncan, 1980; Tapsall, 1987). Previous studies proposed that the 78-kD protein encoded by the cylE gene located in the cyl operon (Fig. 5 A) is the GBS hemolysin, as deletion of the cylE gene abolished hemolytic activity and expression of cylE increased hemolysis in E. coli (Pritzlaff et al., 2001). However, Pritzlaff et al. (2001) also reported that in E. coli expressing CylE, they could not detect the CylE protein in secreted/extracellular fractions and also could not extract hemolytic activity from the bacterial cells using starch, BSA, or tween, characteristic of the GBS hemolysin. These observations suggest that increased hemolytic activity in E. coli may not be a result of the GBS hemolysin. Also noteworthy is that CylE has 43 rare codons that are not efficiently translated in E. coli (i.e., 24 Arg [AGG, AGA, CGA], 5 Leu [CTA], 11 Ile [ATA], and 3 Pro [CCC]). BLAST searches have revealed no significant homology of CylE to known pore-forming toxins and the protein does not possess any canonical secretion signal (Pritzlaff et al., 2001; Nizet, 2002). Moreover, SDS–PAGE analysis of cell-free extracts of GBS with hemolytic activity did not reveal the presence of any protein (unpublished data). To further confirm that cylE alone is necessary and sufficient for GBS hemolysis, we constructed a GBS strain that lacked all genes of the cyl operon (ΔcylX-K; see Fig. 5 A for operon). This strain is nonhemolytic and, interestingly, complementation with plasmids that constitutively express either CylE or CylABE (which includes the ABC transporter system CylA/B; Gottschalk et al., 2006) failed to restore hemolytic activity (Fig. 5 B). In contrast, these plasmids restored hemolytic activity to the GBS strain lacking only cylE (Fig. 5 B) but were unable to induce hemolysis in E. coli (Fig. 5 C). Consistent with these observations, complementation of GBSΔcovRΔcylE with pCylE restored hemolytic activity

### Table 1. GBS clinical isolates associated with preterm labor and mutations in covR/S locus

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Source</th>
<th>Mutation in covR/S loci</th>
<th>Gestational age at birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amniotic fluid</td>
<td>Deletion of adenine residue at position 658 in covS resulting in truncation of CovS at amino acid 220 (CovS 220Stop)</td>
<td>30 wk</td>
</tr>
<tr>
<td>1</td>
<td>Chorioamnion</td>
<td>Same as above i.e. CovS 220Stop</td>
<td>30 wk</td>
</tr>
<tr>
<td>2</td>
<td>Amniotic fluid</td>
<td>Deletion of 4 nucleotides ‘ATTT’ spanning -110 to -107 upstream to the ATG start codon in the promoter region of covR/S</td>
<td>34 wk</td>
</tr>
<tr>
<td>3</td>
<td>Amniotic fluid</td>
<td>Substitution of adenine instead of guanine resulting in amino acid substitution from valine to methionine in CovS (CovS V343M)</td>
<td>26 wk</td>
</tr>
<tr>
<td>3</td>
<td>Blood</td>
<td>CovS V343M</td>
<td>26 wk</td>
</tr>
<tr>
<td>4</td>
<td>Chorioamnion</td>
<td>CovS V343M</td>
<td>36 wk</td>
</tr>
<tr>
<td>5</td>
<td>Chorioamnion</td>
<td>None</td>
<td>36 wk</td>
</tr>
<tr>
<td>6</td>
<td>Chorioamnion</td>
<td>None</td>
<td>28 wk</td>
</tr>
</tbody>
</table>

aIn some patients, GBS were isolated from two different locations, and both these isolates had the same mutation in covR/S.
bOnly nonsynonymous changes are reported.
cAll women enrolled in the study had preterm labor with intact membranes at ≤34 wk of gestation.
Figure 4. GBS clinical isolates from women in preterm labor exhibit increased hemolysis and some are associated with CovR/S mutations. (A) qRT-PCR on RNA isolated from log phase GBS (OD600nm = 0.3). Data are normalized to relative expression of the housekeeping gene rpsL and are the mean and SD from five independent biological replicates performed in triplicate (n = 5; *, P = 0.03, Wilcoxon matched-pairs rank test). (B) Hemolytic activity of GBS clinical isolates associated with preterm labor. A representative image from one of three independent experimental replicates is shown. (C) Single colonies were patched and streaked on blood agar. Hemolysis of GBS WT, ΔcovS, ΔcovR, and complementing clones including plasmids encoding CovS220stop and CovSV343M. Hemolysis of GBS with the pCovR promoter deletion compared with WT. Complementation of GBSΔcovRΔcylE with pCylE on hemolytic activity. A representative image from one of three independent experimental replicates is shown. (D) Invasiveness of GBS ΔcovS to hAEC compared with WT and ΔcovR. Complementation of ΔcovS and ΔcovR on amniotic invasion and effect of pCylE on amniotic epithelial invasion of ΔcovRΔcylE. Data shown are the mean and SD obtained using hAEC from three independent placentas, performed in triplicate (n = 3; ****, P < 0.0001; ***, P = 0.0006, Student’s t test, error bars ± SD). (E) Barrier disruption of amniotic epithelium by GBS WT, ΔcovS, ΔcovR, and ΔcovRΔcylE. Complementation of GBSΔcovRΔcylE with pCylE on amniotic barrier disruption. A representative image from one of three independent experimental replicates is shown.

to levels greater than WT (Fig. 4 C) due to derepression of the other cyl operon genes in the absence of CovR (Lamy et al., 2004; Jiang et al., 2008; Lembo et al., 2010). Collectively, these observations indicate that although necessary, CylE and its putative transporter CylA/B are not sufficient for GBS hemolytic activity.
The functional basis of GBS hemolytic activity is the ornithine rhamnolipid pigment.

Although the hemolytic phenotype of GBS anomalously correlates with pigmentation, where nonhemolytic strains are non-pigmented and hemolytic strains are pigmented (Nizet et al., 1996; Spellerberg et al., 2000; Pritzlaff et al., 2001; also see Fig. 5 D), this link is not understood. The pigment was recently described to be an ornithine rhamnolipid known as granadaene (Rosa-Fraile et al., 2006). Like hemolytic activity, pigment biosynthesis in GBS requires the 12-gene cyl operon (Spellerberg et al., 2000; Pritzlaff et al., 2001; Fig. 5 A) and several genes in this operon (cylD, cylG, cylII, cylK, cylZ, and...
activity to GBSΔcylX-K, the absence of protein in the hemolytic extracts, and observations that disruption of several other cyl genes such as cylD, acpC, cylZ, cylA/B, and cylK abolished hemolytic activity (Spellerberg et al., 1999; Pritzlaff et al., 2001; Gottschalk et al., 2006; Forquin et al., 2007), we hypothesized that hemolytic activity of GBS may be due to the ornithine rhamnolipid pigment and not due to the CylE protein. To test this hypothesis, we extracted the pigment from WT GBS using DMSO containing 0.1% TFA as previously described (Rosa-Fraile et al., 2006). Subsequently, the pigment was purified by gel filtration column chromatography designed for

Figure 6. Proposed biosynthetic pathway for the GBS pigment granadaene. (A and B) Predicted functions of the cyl operon proteins and proposed biosynthetic pathway for synthesis of the GBS pigment using acetyl-CoA, malonyl-CoA, ornithine, and rhamnose. CylD conjugates the elongating malonyl CoA units to the acyl carrier protein (ACP) AcpC. CylI links the malonyl CoA to an initial fatty acid–ACP complex, beginning the fatty acid biosynthesis–like pathway. CylG reduces the 3-keto group to a hydroxyl group, which is further reduced to an alkene by CylZ. The cyl operon lacks an enoyl-ACP reductase thereby eliminating the final reduction of the alkene to an alkane. The unsaturated fatty acid serves as a substrate for further elongation by CylI, accounting for the large degree of unsaturation in the pigment. After 13 total rounds of elongation, the fatty acid is conjugated to ornithine by CylE, and glycosylated by CylJ, CylX, CylF, and CylK likely function upstream or downstream of this pathway. CylX is homologous to a component of the acetyl-CoA carboxylase, which generates malonyl-CoA. CylF is an aminomethyltransferase, likely involved in production of the methylated derivative seen in the mass spectrum (Fig. S2). CylK is a putative phosphopantetheinyl transferase, which is involved in the activation of acyl carrier proteins. Although GBS has a separate fatty acid biosynthesis (fab) operon, deletion of genes in the cyl operon (ΔcylX-K, Fig. 5B) abolishes pigment biosynthesis suggesting that the fab and cyl operons are not functionally redundant.

acpC) encode enzymes catalyzing different steps in fatty acid biosynthesis (Fig. 6 A). The cyl operon also encodes a glycosyltransferase (CylJ), an aminomethyltransferase (CylF), acetyl CoA carboxylase (CylX), and an ABC transporter (CylA/B). Sequence profile analysis of CylE revealed an N-terminal domain of the acyltransferase superfamily known to catalyze several amidoligase reactions (Fig. S1). Using these predicted homologies, a pathway for GBS pigment biosynthesis that requires most genes of the cyl operon is proposed (Fig. 6 B).

Based on the tight link between hemolysin and pigment production, the failure of cylE or cylABE to restore hemolytic
selective purification of small molecules (<5 kD) using a Sepha-
dex LH-20 column and DMSO:0.1% TFA as the mobile phase (Rosa-Fraile et al., 2006). Although soluble in DMSO:0.1% TFA, the pigment was nonhemolytic in this solvent as ob-
served previously (Rosa-Fraile et al., 2006). Because traditional isolation of GBS hemolytic extracts requires a large carrier molecule such as starch (Marchlewicz and Duncan, 1980; Tapsall and Phillips, 1991), we reasoned that addition of starch to the purified pigment may be required for functional activity. Thus, we dissolved purified pigment in DMSO containing 0.1% TFA and 20% starch (DTS). As a control, the pigment extraction procedure was performed on the nonhemolytic and nonpigmented Δcy/E strain. We then examined hemolytic activity of purified pigment using two methods that involved lysis of RBCs. First, twofold dilutions of pigment were used to examine lysis of human RBCs (hRBCs) using the

Figure 7. The functional basis of GBS hemolytic activity is the pigment. (A) Pigment was added to hRBCs in twofold serial dilutions from 25 to 0.024 µM. As controls, equivalent amounts of sample from Δcy/E or DTS were added to hRBC. The data shown are the mean and SD from three independent pigment preparations, performed in triplicate (n = 3, P < 0.0001 Student’s t test, error bars ± SD). (B) Varying amounts of purified GBS pigment (0.33, 0.67, and 1.34 µg) was spotted on sheep blood agar plates and incubated overnight at 37°C. Equivalent amounts (2, 1, and 0.5 µl) of purified extract from GBSΔcy/E and DTS were spotted as controls. A representative image from one of three independent experimental replicates is shown. (C) Scanning electron micrographs showing hRBC membrane morphology after a brief (8 min) exposure to GBS pigment (12.5 µM) or an equal amount of control (buffer or Δcy/E extract). The experiment was performed twice using independent pigment preparations. (D) For proteinase K (PK) treatment of pigment before hemolytic assays, pigment, and control Δcy/E samples in DTS were lyophilized and digested in the presence and absence of proteinase K. The data shown are the mean and SD from three independent pigment preparations, performed in triplicate (n = 3, P ≥ 0.9 Student’s t test, error bars ± SD).
resuspended in proteinase K buffer, and digested in the presence and absence of proteinase K (see Materials and methods for details). Subsequently, hemolytic titer assays were performed on all samples including controls. The results shown in Fig. 7D indicate that pigment treated with proteinase K had similar hemolytic properties compared with pigment not treated with proteinase K (P ≥ 0.9). We further performed Fourier transform ion cyclotron resonance tandem mass spectrometry and nuclear magnetic resonance (NMR) on the purified pigment and control \( \Delta \text{cylE} \) extract (Figs. S2–S4). A comparison of the MS spectra revealed that peaks at 677.38 \( \text{m/z} \) and a methylated derivative at 691.46 \( \text{m/z} \), characteristic of the GBS pigment, are uniquely present in the purified pigment and not in control \( \Delta \text{cylE} \) (Fig. S2). \(^1\)H NMR and correlation spectroscopy (COSY) confirms the structure of the pigment and its presence in the pigment from WT (Fig. S3, A and B) and not in the \( \Delta \text{cylE} \) control (Fig. S4). Also, SDS-PAGE analysis of purified pigment followed by Ruby staining did not reveal the presence of any protein and LC-MS/MS analysis of tryptic digests of purified pigment only identified peptides corresponding to trypsin and common contaminants in these analyses, i.e., keratin (unpublished data). Collectively, these results indicate that the hemolytic molecule purified from GBS is not a protein, but the ornithine rhamnolipid previously described as granadaene (Rosa-Fraile et al., 2006; Vanberg et al., 2007).

The hemolytic activity of the ornithine rhamnolipid is not sensitive to proteinase K

To determine if the hemolytic activity observed with purified pigment could be attributed to a protein toxin, we performed proteinase K digestion of the purified pigment before hemolytic assays. For proteinase K digestion, GBS pigment and control \( \Delta \text{cylE} \) extract previously dissolved in DTS was lyophilized, resuspended in proteinase K buffer, and digested in the presence and absence of proteinase K (see Materials and methods for details). Subsequently, hemolytic titer assays were performed on all samples including controls. The results shown in Fig. 7D indicate that pigment treated with proteinase K had similar hemolytic properties compared with pigment not treated with proteinase K (P ≥ 0.9). We further performed Fourier transform ion cyclotron resonance tandem mass spectrometry and nuclear magnetic resonance (NMR) on the purified pigment and control \( \Delta \text{cylE} \) extract (Figs. S2–S4). A comparison of the MS spectra revealed that peaks at 677.38 \( \text{m/z} \) and a methylated derivative at 691.46 \( \text{m/z} \), characteristic of the GBS pigment, are uniquely present in the purified pigment and not in control \( \Delta \text{cylE} \) (Fig. S2). \(^1\)H NMR and correlation spectroscopy (COSY) confirms the structure of the pigment and its presence in the pigment from WT (Fig. S3, A and B) and not in the \( \Delta \text{cylE} \) control (Fig. S4). Also, SDS-PAGE analysis of purified pigment followed by Ruby staining did not reveal the presence of any protein and LC-MS/MS analysis of tryptic digests of purified pigment only identified peptides corresponding to trypsin and common contaminants in these analyses, i.e., keratin (unpublished data). Collectively, these results indicate that the hemolytic molecule purified from GBS is not a protein, but the ornithine rhamnolipid previously described as granadaene (Rosa-Fraile et al., 2006; Vanberg et al., 2007).
The GBS pigment is cytotoxic to hAECs

To determine if the purified pigment has cytotoxic properties attributed to the function of the previously elusive GBS hemolysin, varying concentrations of purified pigment or buffer and ΔcylE controls were added to hAEC followed by trypan blue staining. These results indicate that the purified pigment possesses cytolytic properties, as indicated by increased trypan blue staining indicative of dead cells in the presence of pigment in contrast to buffer or ΔcylE controls (Fig. 8, A and B, P < 0.005). ELISA assays for cytokines indicated that the purified pigment was unable to induce an inflammatory response in hAEC (unpublished data). These results can be expected because GBS hemolysin/pigment is associated with the bacterial cell surface and is not secreted. These observations indicate that although the purified pigment may induce pore formation similar to other rhamnolipids (Kawai et al., 1982; Sánchez et al., 2010) and polyenes (Knopik–Skroocka and Bielawski, 2002), exposure of host cells to bacterial cell surface associated hemolysin is required for induction of the inflammatory response.

DISCUSSION

This work provides novel evidence that elevated expression of a virulence factor promotes GBS penetration of the amniotic cavity, a critical step in the pathway to preterm birth and fetal injury. Currently, there is no effective vaccine to prevent maternal to infant transmission of GBS. Knowledge of virulence factors associated with preterm birth and neonatal infections will allow for a more informed approach toward strategies for prevention of GBS infections. We have demonstrated that hemolysin promotes GBS invasion of placental cells and that hyper-hemolytic strains are more proficient in disruption of the amniotic barrier and penetration of placental membranes. We predict that environmental changes in the lower genital tract (e.g., neutral pH; Santi et al., 2009) may be sensed by sensor kinases, such as CovS, to alleviate CovR repression, thus triggering an increase in hemolysin expression that mediates ascending GBS infection. Alternatively, mutations in hemolysin regulators such as covR/S that potentially arise while GBS is in a commensal niche (e.g., during vaginal colonization) may promote penetration of chorioamnion and invasion of the amniotic cavity. Consistent with this hypothesis, hyper-hemolytic GBS with covR/S mutations were isolated from the amniotic fluid, chorioamnion, and cord blood of women who delivered preterm. However, GBS strains that had no mutations in the covR/S loci were also recovered from women in preterm labor. Although repression of hemolysin by the CovR/S two component system has been demonstrated in several GBS strains (e.g. A909, COH1, NEM316, 515, 2603v/r, and NCTC10/84; Lamy et al., 2004; Jiang et al., 2005, 2008; Rajagopal et al., 2006; Lin et al., 2009; Santi et al., 2009; Firon et al., 2013) and covR/S and cyl genes are conserved among all sequenced GBS strains (Glaser et al., 2002; Tettelin et al., 2005), it is likely that additional strain-specific regulators also influence the expression of this important virulence factor. Regulators such as the sensor kinase Stk1 (Rajagopal et al., 2006; Lin et al., 2009) and an Abi-domain protein Abx1 (Firon et al., 2013) influence the expression of cyl genes through their interaction with CovR and CovS, respectively. As strains lacking Stk1 or Abx1 exhibit decreased hemolytic activity (Rajagopal et al., 2006; Lin et al., 2009; Firon et al., 2013), increased hemolytic activity observed in the clinical isolates cannot be attributed to the loss of Stk1 or Abx1 and DNA sequence analysis also did not indicate the presence of any mutations in these genes (unpublished data). The complexity of hemolysin regulation is emerging and will be beneficial for a greater understanding of GBS infections associated with preterm premature rupture of membranes (PPROM), preterm delivery (PTD), and neonatal sepsis.

For the first time, we describe the biochemical nature of the GBS hemolysin and elucidate the connection between hemolysis and pigmentation. We show that CylE is necessary but not sufficient for GBS hemolysis and that the ornithine rhamnolipid pigment is hemolytic and cytotoxic. Although a previous study suggested that the GBS hemolysin is likely to be a protein as hemolytic activity diminished due to protease treatment (Marchlewicz and Duncan, 1981), three proteins, including a glycoprotein, copurified with the hemolysin in these prior studies. Given the promiscuity of stabilizers used by the GBS hemolysin (starch, tween, and BSA; Marchlewicz and Duncan, 1980; Tapsall, 1987), we predict that protease digestion of the proteins which copurified with the GBS pigment/ hemolysin may have destabilized the pigment leading to the erroneous conclusion that the GBS hemolysin is a protein.

Our finding that an ornithine rhamnolipid is the hemolysin has important implications for GBS disease pathogenesis. As the GBS hemolysin/pigment is a surface-associated toxin, we predict that the cytotoxic effects are primarily extracellular but that the toxin also mediates host cell lysis when bacteria are internalized into host cells. These cytotoxic properties contribute to barrier failure, thus promoting bacterial dissemination within the host. Hemolysin GBS also induce a strong immune response (Fig. 2, A and B; Fig. 3 C), which plays an important role in its disease pathogenesis. However, specific host immune pathways that respond to this virulence factor remain undefined (Nizet et al., 1996; Doran et al., 2002; Henneke et al., 2002; Lembo et al., 2010; Bebien et al., 2012). With the identification, purification, and biochemical characterization of the GBS hemolysin as the ornithine rhamnolipid pigment, key mechanisms of host immune activation can now be addressed. Such studies will provide critical insight necessary for preventive strategies against GBS infections. Although vaccines that target surface proteins was shown to provide protection against GBS in mouse models of infection (Mainone et al., 2005), protein-based vaccines cannot neutralize the effect of this lipid toxin. A previous attempt to raise anti-sera to crude extracts of the GBS hemolysin was unsuccessful (Dal and Monteil, 1983) and is consistent with the notion that lipids typically do not elicit an antibody response. However, therapeutic measures designed to inhibit biosynthesis of the ornithine rhamnolipid pigment or its function may prove effective.

Although ornithine–containing lipids are widely known in bacteria (Geiger et al., 2010) and a few have hemolytic or hemagglutinating properties (Kawai et al., 1982; Häussler et al.,
1998; Sánchez et al., 2010), their role in virulence of bacterial pathogens is not fully appreciated. Orthologs of the cyl operon are present in other bacterial species including opportunistic human and insect pathogens such as Paenibacillus larvae, Paenibacillus thuringiensis, Paenibacillus larvae, and actinobacteria such as Actinomyces viscosus, Kitasatorpora setae, Arthrobacter species, and Propionibacterium species (Fig. 9). In these organisms, the core cyl operon containing genes for fatty acid and ornithine biosynthesis and their amidoligation are strongly conserved. Also, P. jensenii is described to produce a pigment similar to the GBS granadae (Vanberg et al., 2007). Given their ubiquitous nature, our findings have significant implication in the classification of bacteria that encode pigments with hemo-lytic and cytolytic properties. In summary, our work shows that GBS virulence and its transition from commensal to invasive niches hinges on its ability to regulate the expression of a key virulence factor which we describe is a hemolytic ornithine lipid.

MATERIALS AND METHODS

Human subjects. Written informed patient consent for donation of normal, term placentas immediately after cesarean delivery from women without labor was obtained with approval from the University of Washington Institutional Review Board (protocol #34004). GBS clinical isolates from amniotic fluid, chorioamnion, and/or cord blood were obtained from women enrolled with preterm labor and intact membranes at ≤34 wk gestation at the University of Washington Medical Center, Swedish Medical Center, and Virginia Mason Medical Center (Seattle, Washington) between June 25, 1991 and June 30, 1997. This cohort was previously described (Jiang et al., 2005). Routine cultures of GBS were grown in Tryptic Soy Broth (TSB; Difco Laboratories) at 37°C in 5% CO2, and routine cultures of E. coli were performed in Luria-Bertani Broth (LB; Difco Laboratories) at 37°C. Cell growth was monitored at 600 nm. Antibiotics were added at the following concentrations when necessary: for GBS, 1 µg/ml erythromycin, 300 µg/ml spectinomycin, 1,000 µg/ml kanamycin, and 2.5–5 µg/ml chloramphenicol; for E. coli, 300 µg/ml erythromycin, 50 µg/ml spectinomycin, 50 µg/ml kanamycin, and 10 µg/ml chloramphenicol. Antibiotics and other chemicals were purchased from Sigma-Aldrich. Cell culture media was purchased from Mediatech Inc. All GBS mutants used in this study had similar growth rates compared with isogenic WT in the media used for cell culture and ex vivo experiments (DMEM containing 1% FCS). Restriction enzymes were purchased from Fermentas, and primers were purchased from Sigma-Aldrich. RNA isolation and qRT-PCR for analysis of GBS gene expression was performed as described previously (Lembo et al., 2010). GBS pictures shown on Red Blood Agar and Granada Media were captured using a digital SLR camera (EOS Rebel XSi 12.2MP; Cannon) with an 18–55 mm zoom lens and processed using Photoshop CS2 (version 9; Adobe) and compiled using Canvas 9 (version 9.0.4; Deneba).

Construction of GBS ΔcylX-K and complementing plasmids. Approximately 1 kb of DNA located upstream of cylX and 1 kb of DNA located downstream of cylK were amplified using high-fidelity PCR (Invitrogen) and primer pairs dCylXupF and dCylXupR, and dCylKdownF and dCylKdownR, respectively. The gene conferring kanamycin (Ω km-2) resistance was also amplified using high-fidelity PCR from pCIV2 (Okada et al., 1993) for allelic replacement of cylX-K, using primers dCylXupF and dCylXupR. Subsequently, strand overlap extension PCR (Horton, 1995) was performed to introduce the antibiotic resistance gene (Ω km-2) between the flanking regions of cylX-K described above. The PCR fragment was then ligated into the temperature-sensitive vector pHY304 (Chaffin et al., 2000) and the resulting plasmid was electroporated into GBS WT as described previously (Rajagopal et al., 2003). Selection and screening for the double crossover mutant was performed as previously described (Rajagopal et al., 2003). PCR was used to verify the presence of Ω km-2 and the absence of cylX-K. The genes encoding cylE, cylABE, covR, and covS were amplified using high-fidelity PCR using primer pairs CylDEF and CylER, CylABEF and CylABER, CovRF and CovSR, and CovSF and CovSR, respectively. The PCR fragments were digested with restriction enzymes present on the primer sequences and then ligated into the following plasmids: pAC23, pCUT26, pCUT32, and pCUT52. The resulting plasmids were then transformed into GBS ΔcylX-K and complementing plasmids.
cloned into the multiple cloning site of the GBS complementation vector pDC123 downstream to the constitutive promoter as described previously (Chaffin and Rubens, 1998; Rajagopal et al., 2003). The complementing plasmids were then electroporated into GBS ΔcytE, ΔcytX-K, ΔowR, and ΔowM using methods previously described (Rajagopal et al., 2003). Site-directed mutants were generated using the QuikChange Site-Directed mutagenesis kit (QIAGEN) with the complementing pSsPlasmid as the template and primers CovS220F & CovS220R for pCovSV2Stop and primers CovSV343MF & CovSV343MR for pCovSV343M. GBS with chromosomal mutations in CovR/S loci was obtained using methods described previously (Lin et al., 2009). DNA sequencing was performed to confirm the presence of the desired mutations.

**Derivation of human amniotic epithelium.** Primary hAECs were isolated and cultured from normal term placentas obtained immediately after cesarean delivery from women without labor as previously described (Sun et al., 2003). In brief, the amnion was peeled from the chorion, and the amnion tissue was washed with PBS and digested with trypsin (Worthington Biochemical Corp.) and DNase (Sigma-Aldrich) as described previously (Sun et al., 2003). Subsequently, the trypsin digestion media was centrifuged and cell pellets were resuspended in DMEM and loaded onto pre-prepared discontinuous Percoll (GE Healthcare) gradients (5, 20, 40, and 60%, respectively), and the gradients were centrifuged as previously described (Sun et al., 2003). A single band of cells at ~20% Percoll concentration was collected, diluted in media to a density of 10⁶ cells/ml, and cultured as previously described (Sun et al., 2003).

**Infection assays.** Three to four independent placentas were used and all experiments were performed in triplicate. Adherence and invasion of GBS WT COH1 or 5009 and isogenic ΔcytE, ΔowR, ΔowM, and ΔowMΔcytE mutants to hAEC were performed as previously described (Nizet et al., 1997; Winram et al., 1998). In brief, GBS strains grown to mid-log phase (~10⁹ CFU/ml; OD₆₀₀nm = 0.3) were washed in PBS, resuspended in DMEM with 10% FBS, and used to infect hAEC monolayers at a multiplicity of infection of 1. For adherence assays, the infection was performed for a period of 2 h after which the bacteria were enumerated using methods previously described (Nizet et al., 1997; Winram et al., 1998). For invasion assays, 100 µg/ml gentamicin and 5 µg/ml penicillin was added to each well at 2 h after infection and the plates were incubated for an additional 2 h to kill extracellular and surface-adherent bacteria; subsequently, intracellular bacteria were released using trypsin-EDTA (Gibco) and Triton X-100 and bacteria were serially diluted and enumerated as previously described (Nizet et al., 1997; Winram et al., 1998).

**Expression of inflammatory mediators.** Three independent placentas were used and all experiments were performed in triplicate. hAECs were cultured, washed, and infected with GBS strains as described above. Subsequently, total RNA was isolated from hAEC monolayers using the RNeasy miniprep kit (QIAGEN) according to the manufacturer’s protocol and digested with DNase I to remove contaminating genomic DNA. Expression of inflammatory mediators in human amniotic epithelium was determined using qRT-PCR as described previously (Lembo et al., 2010). Supernatants from GBS-infected hAEC were collected, centrifuged to remove bacteria, and Luminex bead assays (Millipore) were performed using methods described by the manufacturer to evaluate cytokine levels.

**Western blots.** hAECs were grown to confluence and infected with GBS at a multiplicity of infection of 1 for a period of 4 h. Uninfected cells were included as controls. Subsequently, hAECs were washed, digested with trypsin, and centrifuged. The NE-PER kit (Thermo Fisher Scientific) was used for stepwise separation and preparation of cytoplasmic and nuclear extracts from infected hAEC as per manufacturer’s instructions. Equal amounts (10 µg) of cytoplasmic and nuclear extracts were subjected to 7.5% SDS-PAGE followed by Western blotting. The membrane was blocked in 1:1 Odyssey blocking buffer (Li-Cor Biosciences) in PBS and then incubated at 4°C overnight with a 1:200 dilution of primary NF-κB p65 antibody (Santa Cruz Biotechnology). Secondary antibody Alexa Fluor 680 anti–mouse (Life Technologies) was added at a 1:1,000 dilution. After several washes, the membrane was visualized with the Odyssey Li-Cor infrared imager (Li-Cor Biosciences).

**Barrier integrity analysis.** Changes in transepithelial electrical resistance across hAEC monolayers were measured in real time using ECIS (Giaever and Keece, 1993) with an ECIS ZTheta Instrument and 8W10E+ arrays (Applied BioPhysics). hAEC monolayers were established on gold-plated electrodes in 8-well array slides attached to a computer-operated sensing apparatus to allow measurements in real time. Monolayers were then infected with the GBS strains at 10⁵ CFU/ml and the system measured the cell membrane capacitance (Cm), the resistance from the cell-electrode interaction (α), and the barrier function properties of the cell monolayer (Rb). Deconvolution of the overall ECIS signal into these parameters is performed by the ECIS software by fitting the mathematical model derived by Giaever and Keece (1991) to the experimental data by least-square optimization procedures. Uninfected wells served as controls for background levels of electrical resistance. Data are represented as a change in resistance as a proportion of the control over time as described previously (Lembo et al., 2010). A representative of three independent experiments is shown.

**Ex vivo placental model.** Intact chorioamnionic membranes obtained immediately after normal, cesarean delivery were rinsed in sterile saline solution and placed over an inverted, upper chamber of a Transwell System (Corning Inc.) from which the original polycarbonate membrane was previously removed as described previously (Zagay-Cleavilla et al., 2007). Sterile silicone rubber rings were used to hold the placental membranes in place. Thus, when the transwell is inserted, the choriodedecida faces the upper chamber and the amniotic epithelium faces the lower chamber as previously described (Zagay-Cleavilla et al., 2007). Care was taken to during the entire procedure so that there were no tears or rips on the placental membranes mounted on the transwell. The explants were stabilized in media for a period of 48 h. Six independent placenta/chorioamnionic membranes were used in these assays. Uninfected placenta was also included as a control in each experiment. For infection of chorionamnion, GBS strains were grown to an OD₆₀₀nm of 0.3, washed twice in PBS, and ~10⁵ CFU in a final volume of 1 ml DMEM was added to the upper chamber. At 24 h after infection, media (DMEM) from the lower chamber was removed and processed for bacterial enumeration and expression of cytokines. For bacterial enumeration, aliquots of the media were serially diluted and plated on TSA plates. For analysis of inflammatory mediators, aliquots of media were analyzed using ELISA or the Luminex bead assay as described by the manufacturer (R&D Systems or Millipore). For microscopy, infected chorioamnionic membranes were fixed in 10% phosphate buffered formalin at 4°C overnight and stored in 70% ethanol. The membranes were subsequently embedded in paraffin, sectioned, and stained using the Gram-Twost stain as previously described (Stevens and Bancroft, 1977). Images were captured in bright field using the DM4000B Fluorescent upright microscope (Leica) under 10, 40, and 100x magnifications. The microscope was attached to a DFC310FX camera (Leica) and the acquisition software used was the Leica application suite (version 4.0.0). A representative image from experiments with six independent placentas with similar results is shown. As controls for bacterial migration, transwells were also mounted with either chorion alone or amnion alone. In brief, the amnion was peeled from the chorion and individual membranes were mounted over the transwell such that their orientation was maintained, i.e., the inner chamber represented the choriodedecida side for chorionic transwells and amniotic mesoderm for amniotic transwells. Infection with GBS strains was performed as described above.

**Purification and characterization of the ornithine rhamnolipid pigment.** GBS pigment was purified as previously described (Rosa-Fraile et al., 2006) with some modifications. In brief, WT GBS were grown at 37°C in New Granada Media (de la Rosa et al., 1992) until the broth turned red (48–72 h). Bacterial cells were pelleted, washed three times with distilled water and twice with DMSO. The cell pellet was then resuspended in DMSO:0.1% TFA overnight to extract the pigment, cell debris was pelleted, and the supernatant containing the pigment was saved. The above
process was repeated until the supernatant obtained from GBS cells was clear. Pigment was then precipitated by addition of 25% NH4OH to a final concentration of 0.25% as previously described (Vanberg et al., 2007). Precipitated pigment was washed three times with HPLC grade water and twice in DMSO, redissolved in DMSO-0.1%TFA, and purified using a Sephadex LH-20 (GE Healthcare) column as previously described (Rosa-Fraile et al., 2006; Vanberg et al., 2007). Fractions containing purified pigment were pooled and precipitated with NH4OH (Scientific Products) as described above, washed three times with HPLC grade water, twice with DMSO, and lyophilized. As a control, GBSΔerylE was also grown in New Granada Media and pigment extraction protocol was followed as described above. For NMR analysis, purified pigment or control ΔerylE samples were resuspended in DMSO-d6, 0.1% d-TFA (Sigma-Aldrich), 1H, 13C, 1H-COSY NMR experiments were performed at 298K on a Bruker AV-500 NMR Spectrometer. Residual DMSO-d6 was used to calibrate chemical shifts. For MS experiments, lyophilized pigment or control ΔerylE samples were dissolved in DMSO-0.1%TFA and analyzed by Fourier Transform Ion Cyclotron Resonance mass spectrometry on a Bruker AutoFlex APEX Qe 47e instrument. For fermentation, lyophilized pigment or control ΔerylE extract was dissolved in DTS to a final concentration of 1 mM. The samples were incubated overnight at room temperature in the dark before use.

Hemolytic titer assays was performed using methods described with some modifications (Nizet et al., 1996). In brief, twofold serial dilutions of purified pigment or control ΔerylE extract in DTS was performed in PBS + 0.2% glucose in a final volume of 100 µl. These samples were then incubated with 100 µl of heparin-treated hRBCs (1%) in 96-well plates at 37°C for 1 h, after which the plates were spun for 4 min at 3,000 × g to pellet unlysed hRBC. The supernatants were transferred to a replica 96-well plate and hemoglobin release was measured by recording the absorbance at 420 nm. Positive and negative controls were also included. For MS experiments, lyophilized pigment or control ΔerylE samples were dissolved in DMSO-0.1%TFA and analyzed by Fourier Transform Ion Cyclotron Resonance mass spectrometry on a Bruker AutoFlex APEX Qe 47e instrument. For fermentation, lyophilized pigment or ΔerylE extract was dissolved in DTS to a final concentration of 1 mM. The samples were incubated overnight at room temperature in the dark before use.

Scanning electron microscopy. Erythrocytes from 0.5 ml of human blood was centrifuged, washed twice with PBS, and resuspended in 5 ml PBS. Erythrocytes were then treated with 12.5 µM of pigment or an equivalent amount of controls (DTS or ΔerylE extract) for 8 min at 37°C. Subsequently, the samples were centrifuged at 4°C and the supernatant was discarded. The pellet was resuspended in 0.5 ml of 1/2 Karnovsky’s Fixative and incubated overnight at room temperature. Samples were then prepared for scanning electron microscopy as previously described (Sanchez et al., 2010). Images were captured using a JEOL JSM-6460LV SEM equipped with a JEOL Orion Digital Acquisition System. The experiment was performed twice using three independent preparations of purified pigment.

Cytotoxicity assays. hAECs cultured in 96-well plates (10^5 cells/well) for 24 h were treated with known concentrations of pigment or equivalent amounts of control ΔerylE extract or DTS for a period of 4 h. Subsequently, the media was removed and hAECs were treated with 0.2% trypan blue for 2 min. Cells were washed with PBS and were immediately observed and imaged under a DMi6000B inverted microscope (Leica) and images were captured at 10 and 40× magnifications. The microscopy was equipped with a DFC310 FX (Leica) camera and the acquisition software used was the Leica application suite, version 4.0.0. Data are reported as the mean and SD of five fields per well.

Statistical analysis. The unpaired, two tailed Students t test, Mann-Whitney test, Wilcoxon’s matched pairs rank test was used to estimate differences as appropriate, and a value of P < 0.05 was considered significant. The nonlinear regression analysis was used to estimate EC50 concentration. These tests were performed using Prism for Windows (version 5.0; GraphPad Software).

Online supplemental material. Table S1 lists primers used in this study. Fig. S1 shows the alignment of CylE to known N-acyltransferases. Fig. S2 shows the overlay of mass spectra of purified pigment and control ΔerylE extracts. Fig. S3 indicates 1H chemical shifts and 1H-1H COSY NMR of the purified GBS pigment. NMR of control ΔerylE extract is shown in Fig. S4. Online supplemental material is available at http://www.jcem.org/cgi/content/full/jcem.20122753/DC1.

We thank Dr. Victor Nizet for the GBSΔerylE strain, Dr. David Eschenbach for his input, Dr. Sophia Lannon and Evangelyn Nkwaiakwa for assistance in placenta collection, Dr. Kang Sun for helping with derivation of hAEC, Kathy Agnew for isolation of GBS clinical isolates, Dr. Lisa Cox for help with microscopy, and Nguyen Thao-Binh Tran, Jessica Klein, and Hannah Miller for technical assistance. The authors are grateful to Christian Renken (Applied Biophysics) for assistance with ECD. We thank Dr. Paul Miller and Dale Whittington for assistance with NMR and MS analyses, respectively. We also thank Dr. Toni Kline for her expertise with interpretation of the NMR. Histology was performed using the Histology and Imaging Core at the University of Washington.

This work was supported by funding from the National Institutes of Health (NIH) grants R01AI100989 to L. Rajagopal and K.M. Adams Waldorf, R01AI070749 to L. Rajagopal, and K08AI067910 to K.M. Adams Waldorf. This work was also supported by funding from the March of Dimes grant 21-FY08-562 and 21-FY06-77 to K.M. Adams Waldorf. Support for K. Burnside was provided by the NIH training grant (T32 HD007233-29, PI: Lisa Frenkel). Support for C. Whittboy was provided by the NIH training grant (T32 AI07559, PI: Lee Ann Campbell). L.M. Iyer and L. Aravind are supported by intramural funds from NLM, NIH. The Guyanica Foundation also provided support for this work. We are grateful to the human subjects who participated in these studies. The study on women in preterm labor from where GBS clinical isolates were obtained was funded by R01AI31871.

The authors have no conflicting financial interests.

Submitted: 12 December 2012
Accepted: 1 May 2013

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Bobit, J.R., and W.J. Ledger. 1977. Unrecognized amnionitis and preterm labor from where GBS clinical isolates were obtained was funded by R01AI31871.

The authors have no conflicting financial interests.

Submitted: 12 December 2012
Accepted: 1 May 2013

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Submitted: 12 December 2012
Accepted: 1 May 2013

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