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Short communication

CAMP factor is not essential for systemic virulence of Group B *Streptococcus*

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Abstract

The Gram-positive pathogen Group B *Streptococcus* (GBS) is the leading cause of bacterial pneumonia, sepsis, and meningitis in human newborns. GBS elaborates a pore-forming toxin known as CAMP factor that synergizes with *Staphylococcus aureus* β-toxin, generating a co-hemolytic reaction useful in identification of GBS in the clinical laboratory. To evaluate the indirect evidence implicating CAMP factor in GBS pathogenesis, the *cfb* gene encoding the pore-forming cytotoxin was deleted by precise allelic replacement. The virulence properties of the CAMP factor mutant were then explored by a series of *in vitro* and *in vivo* assays. Compared to wild-type, the isogenic GBS Δ*cfb* mutant demonstrated equivalent phagocyte resistance and endothelial cell invasiveness and also retained full virulence in a mouse model of infection. Our data suggest that CAMP factor expressed in its native context is not essential for systemic virulence of GBS.

Keywords: Bacterial infection; *Streptococcus agalactiae*; Pore-forming toxin; Hemolysin

1. Introduction

Group B *Streptococcus* (GBS) is a major cause of sepsis and meningitis in human neonates. A curious phenomenon of the clinical microbiology laboratory first described by Christie et al. in 1944 [1] involves the lysis of sheep red blood cells by the combined action of *Staphylococcus aureus* β-toxin (sphingomyelinase) and an extracellular product of GBS, commonly known by the acronym CAMP factor after the discoverers. This CAMP factor co-hemolytic reaction later found utility as a diagnostic tool for presumptive identification of GBS [2,3]. In 1988, the complete 226 amino acid sequence of GBS CAMP factor was deduced manually from peptide fragments [4] and its encoding gene (*cfb*) was successfully cloned and expressed in *Escherichia coli* [5]. Subsequent studies have identified CAMP factor homologues in several other bacterial species including groups A, C and G streptococci, *Streptococcus uberis*, *Actinobacillus pleuropneumoniae*, and *Propionibacterium acnes* [6–9].

Elegant functional analyses indicate that GBS CAMP factor oligomerizes to generate discrete pores in target cells, using the carbohydrate core of GPI-anchored proteins as a membrane receptor [10,11]. CAMP factor is often proposed as a GBS virulence factor, but published evidence comprises just two studies applying indirect methods: (a) high concentrations of partially purified preparations of the toxin injected intravenously were lethal to mice or rabbits [12] and (b) mice given sublethal doses of

Abbreviations: GBS, Group B *Streptococcus*; THB, Todd–Hewitt broth; THA, Todd–Hewitt agar; LB, Luria–Bertani broth; cfu, colony-forming units; MOI, multiplicity of infection; *cat*, chloramphenicol acetyltransferase.

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GBS and then treated with seven repeated intravenous injections of purified CAMP factor over the next 9 h developed fatal septicemia [13]. To specifically test the role of *endogenous* CAMP factor production in GBS pathogenicity, we adopted an approach of targeted mutagenesis.

2. Results

2.1. Deletion of *cfb* gene in GBS

The *cfb* gene encoding CAMP factor was deleted in well-characterized virulent serotype III GBS strain COH1 by precise, in-frame allelic replacement with a chloramphenicol acetyltransferase (*cat*) antibiotic resistance marker (Fig. 1A). The isogenic Δ*cfb* mutant lost the ability to generate the co-hemolytic CAMP reaction when streaked adjacent to β-toxin expressing *S. aureus* on sheep's blood agar (Fig. 1B). Complementation studies showed the CAMP phenotype of the Δ*cfb* mutant could be fully restored by reintroduction the wild-type (WT) *cfb* gene on expression plasmid pDCerm [14] (Fig. 1B).

2.2. In vitro phenotypic analysis of WT and isogenic Δ*cfb* mutant GBS

The capacity of GBS to produce systemic infection reflects an ability of the pathogen to resist host innate immune clearance mechanisms. Using published assays [15], we found no differences in the survival kinetics of WT and isogenic Δ*cfb* mutant GBS upon incubation with freshly isolated human whole blood (Fig. 2A) or purified neutrophils (data not shown) from healthy human volunteers. Likewise, no differences were identified in survival of the WT parent strain and Δ*cfb* mutant upon co-incubation with cultured J774 murine macrophages (Fig. 2B). Cathelicidin antimicrobial peptides are important in mammalian defense against invasive streptococcal infection [16], but killing kinetics for murine cathelicidin mCRAMP were similar for WT and Δ*cfb* mutant GBS (Fig. 2C). Invasion of human blood–brain barrier endothelium is a key step in the pathogenesis of GBS meningitis; however, in a standard tissue culture adhesion/invasion assay [17], no differences were observed between the WT and Δ*cfb* mutant in adherence to and invasion of cultured human brain microvascular endothelial cells (hBMEC) (Fig. 2D).

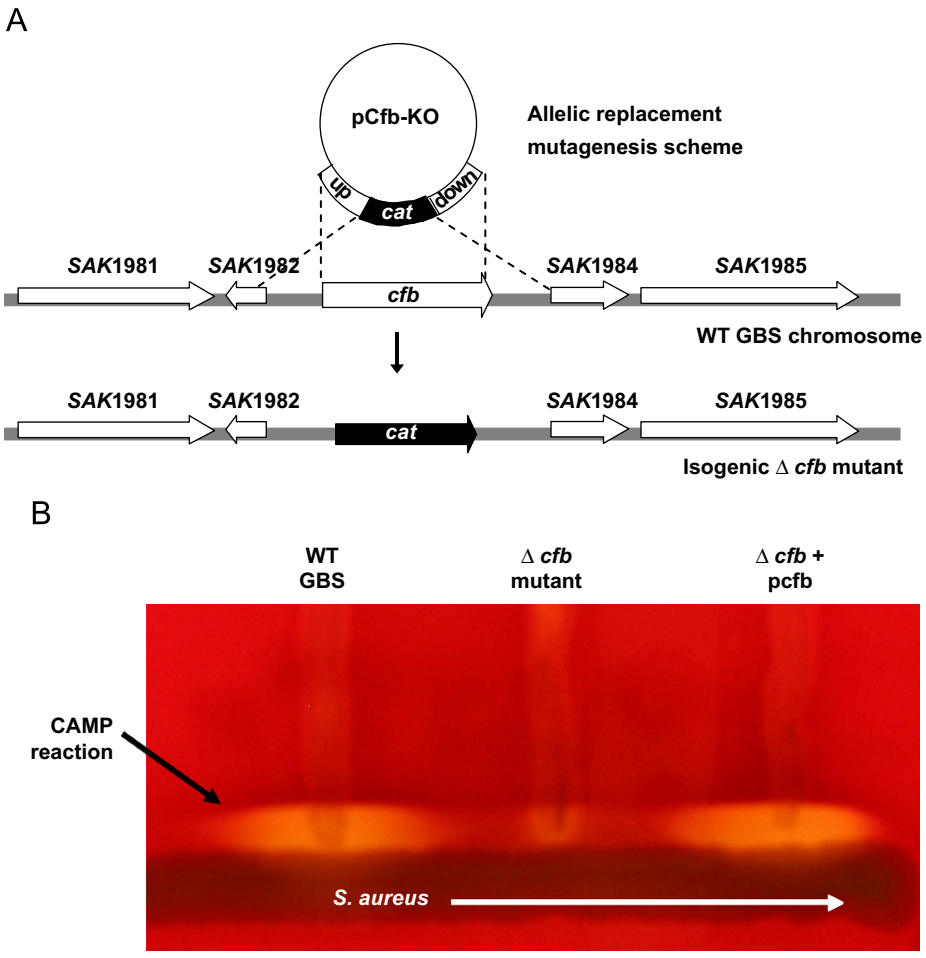

Fig. 1. Mutagenesis of the GBS CAMP factor phenotype. (A) Scheme for targeted allelic replacement of the GBS *cfb* gene (sequences based on genome of GBS strain A909; GenBank CP000114). (B) Loss of co-hemolytic activity of GBS Δ*cfb* mutant when streaked on blood agar media adjacent to β-toxin expressing *Staphylococcus aureus*; CAMP phenotype is restored upon complementation with *cfb* gene on a plasmid vector.

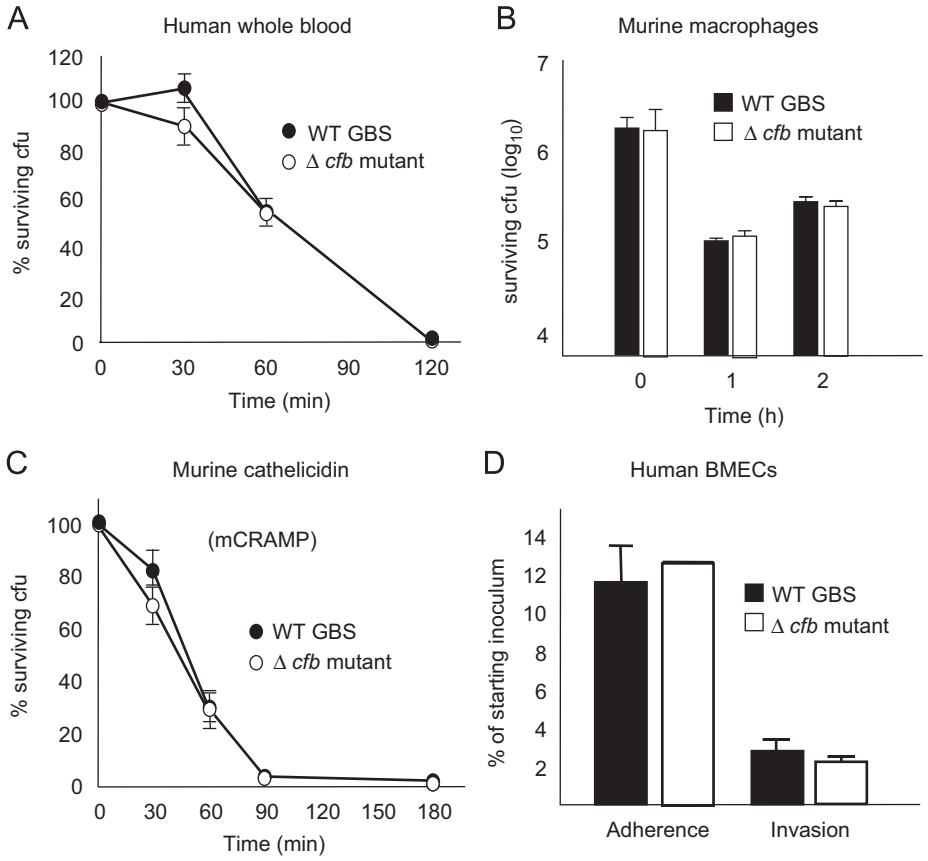

Fig. 2. *In vitro* analyses of GBS Δ*cfb* mutant. The isogenic GBS Δ*cfb* mutant does not differ from the WT parent strain in susceptibility to (A) human whole blood, (B) murine macrophage, or (C) cathelicidin antimicrobial peptide killing. (D) WT GBS and Δ*cfb* mutant are comparable in adherence to and invasion of cultured human brain microvascular endothelial cells.

2.3. Virulence of WT and isogenic Δcfb mutant GBS in mice

To determine the requirement of GBS CAMP factor production for animal virulence, we challenged 8–10 week-old C57BL/6 mice ($n = 10$ per group) with 10^8 cfu of either WT or Δ*cfb* mutant GBS by intraperitoneal injection. Mice were monitored twice daily for mortality, and the Kaplan–Meier survival curves for the two groups were essentially identical (Fig. 3A). Quantitative bacterial cultures from all mice at 4 h and from those surviving at 24 h revealed similar bacterial loads (Fig. 3B).

3. Discussion

GBS elaborates a pore-forming β-hemolysin/cytolysin that promotes phagocyte resistance, host cellular invasion, blood–brain barrier penetration, and virulence in mice [15,18–20]. Tested here using similar *in vitro* and *in vivo* model systems, we were unable to differentiate a significant role for the GBS co-hemolysin CAMP factor in these phenotypes. Our data indicate that despite the reported toxicity of CAMP factor preparations [12,13], the toxin fails to satisfy molecular Koch's postulates [21] as an essential virulence factor in GBS systemic mouse infection.

Immunoglobulin Fc-binding capabilities of purified GBS CAMP factor were previously documented [13], and this phenomenon was originally believed to impact the influence of CAMP factor on septicemia and mortality; however, newer data question the validity of this observation [22]. Nevertheless, conservation of CAMP factor among GBS strains (and in other species listed above) implies an evolutionary benefit of the toxin in the overall context of the bacterium's ecology. Recent evidence reveals expression of the GBS *cfb* gene is differentially regulated through the CsrRS/CovRS two-component system [23,24] and the eukaryotic-type serine/threonine kinase Stk1 [25], which control additional phenotypic features of the pathogen including the β-hemolysin/cytolysin toxin. Future analysis of CAMP factor function will be aided by the availability of the isogenic GBS Δ*cfb* knockout mutant described in this report.

4. Materials and methods

4.1. Bacterial strains

GBS strain COH1 is a well-characterized, highly encapsulated serotype III clinical isolate. *E. coli* strain MC1061 was used for molecular cloning procedures. GBS strains were grown in Todd–Hewitt broth (THB) or agar (Hardy Diagnostics), and *E. coli* were grown in Luria–Bertani broth (LB) or agar (Hardy Diagnostics). Where

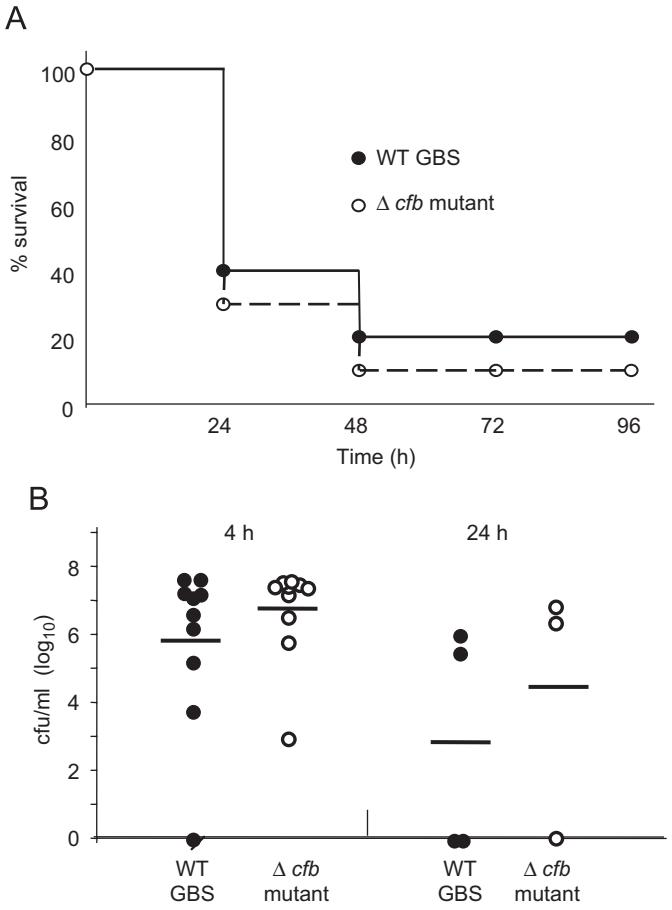

Fig. 3. CAMP factor is not essential for GBS virulence in mice. Following intraperitoneal challenge of C57Bl/6 mice, WT and isogenic Δ*cfb* mutant GBS produce similar mortality profiles (A) and reach equivalent levels of bacterial counts in the blood (B).

required, antibiotic selection utilized 5 μg/ml erythromycin (Em) or 2 μg/ml chloramphenicol (CM) for GBS and 500 μg/ml Em for *E. coli*.

4.2. Allelic replacement mutagenesis and complementation

Our established methods of PCR-based targeting vector construction in temperature-sensitive plasmid pHY304 were utilized [17,26]. Primer sequences were based on the published genome of GBS strain A909 (GenBank CP000114). Briefly, ~500 bp of sequence immediately upstream of *cfb* was from the COH1 chromosome using primers CAMP-UpF 5′-GCTTTTTAAGCGTCTAAACT-ACTTGGTCTAGTATG-3′ and CAMP—UpR 5′-AATG-AATTTCCTCCTTTAAACTAA-3′. Similarly, ~500 bp immediately downstream of *cfb* was amplified from the COH1 chromosome using primers CAMP-DownR 5′-TA-TGGTAGACTACACAGGACATATTTACACT-3′ and CAMP-DownF 5′-TAATATTTGCATTTTTCGTGTG-ATGC-3′. The CAMP-UpR and CAMP-DownF primers were constructed with 25-bp 5′ extensions corresponding to the 5′ and 3′ ends of the *cat* gene, respectively. The amplified PCR products were combined with a 650 bp amplicon of *cat* in a second round of fusion PCR using the CAMP-UpF and CAMP-DownR primers. The resulting PCR amplicon, containing an in-frame substitution of the *cat* gene for *cfb*, was subcloned directly into the temperature-sensitive vector, pHY304. Electroporetic transformation and sequential identification of single and double crossover events in the GBS chromosome by differential antibiotic and temperature selection were completed as described [17,26]. Precise in-frame allelic replacement of *cfb* with *cat* was confirmed by PCR, sequencing and loss of CAMP reaction phenotype. For complementation of the Δ*cfb* mutant, primers CAMP-UpF and CAMP-DownR were used to amplify the WT *cfb* factor gene from the COH1 genome. The fragment was directionally cloned into the expression vector pDCerm [14] and used to transform the Δ*cfb* mutant, and the complemented strain identified by Em resistance and restriction analysis of the purified plasmid.

4.3. In vitro assays

Whole blood and neutrophil killing assays were performed essentially as described [15]. Briefly, whole blood was drawn from consenting healthy volunteers in accordance with UCSD Institutional Review Board policies. Neutrophils were isolated using the Histopaque cell separation system (Sigma). Overnight cultures of GBS were diluted in THB and grown to mid-logarithmic phase ($A_{600} = 0.4 = \sim 10^8$ cfu/ml), washed two times and resuspended in PBS, added to the blood or neutrophils at specified inocula, then incubated at 37 °C on a rotating platform (for whole blood) or in a 96-well plate in a 5% CO_2 incubator (for neutrophils) for up to 3 h, at which point viable GBS cfu were enumerated by plating dilutions on THA. Cultured J774 macrophage were propagated in 24-well plates to $\sim 1 \times 10^6$ cells/well in RPMI + 10% FBS. GBS were added at a multiplicity of infection (MOI) = 0.1 bacteria/cell and incubated for 1 h at 37 °C. Monolayers were washed three times, and media containing gentamicin (100 μg/ml) and penicillin (5 μg/ml) was added for 2 h to kill extracellular bacteria. The monolayers were then washed three times again, cells lysed with Triton X-100, and serial dilutions plated for enumeration of surviving cfu. Bacterial killing by the antimicrobial peptide, CRAMP, was performed essentially as described [16]. Briefly, 6×10^4 cfu bacteria were incubated in a total volume of 200 μl in individual wells of a microtiter plate at 37 °C. Eight micromolar CRAMP was added to each reaction well. Samples were drawn at various time points, and serial dilutions were plated to quantify cfu. The ability of GBS to adhere to and invade cultured hBMEC was performed as previously described [19].

4.4. In vivo mouse infection model

A murine model of systemic infection was used to assess the virulence of the CAMP factor mutant GBS. The *in vivo*

protocol was approved by the University of California, San Diego Animal Subjects Committee. For these studies dilutions of overnight cultures of WT and Δ*cfb* COH1 GBS strains were grown to mid-logarithmic phase. Eight- to ten-week-old C57BL/6 mice ($n = 10$ per group) were then injected intraperitoneally with 10^8 cfu of either bacterial strain and were monitored twice daily for 3 days for mortality. Blood was collected at 4 and 24 h for enumeration of bacterial load. Survival was monitored to 4 days after inoculation. Animals appearing moribund during the study were humanely euthanized.

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