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RESEARCH ARTICLE

# Genetic Basis Underlying the Hyperhemolytic Phenotype of *Streptococcus agalactiae* Strain CNCTC10/84

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**ABSTRACT** *Streptococcus agalactiae* (group B streptococcus [GBS]) is a major cause of infections in newborns, pregnant women, and immunocompromised patients. GBS strain CNCTC10/84 is a clinical isolate that has high virulence in animal models of infection and has been used extensively to study GBS pathogenesis. Two unusual features of this strain are hyperhemolytic activity and hypo-CAMP factor activity. These two phenotypes are typical of GBS strains that are functionally deficient in the CovR-CovS two-component regulatory system. A previous whole-genome sequencing study found that strain CNCTC10/84 has intact *covR* and *covS* regulatory genes. We investigated CovR-CovS regulation in CNCTC10/84 and discovered that a single-nucleotide insertion in a homopolymeric tract in the *covR* promoter region underlies the strong hemolytic activity and weak CAMP activity of this strain. Using isogenic mutant strains, we demonstrate that this single-nucleotide insertion confers significantly decreased expression of *covR* and *covS* and altered expression of CovR-CovS-regulated genes, including that of genes encoding β-hemolysin and CAMP factor. This single-nucleotide insertion also confers significantly increased GBS survival in human whole blood *ex vivo*.

**IMPORTANCE** Group B streptococcus (GBS) is the leading cause of neonatal sepsis, pneumonia, and meningitis. GBS strain CNCTC10/84 is a highly virulent blood isolate that has been used extensively to study GBS pathogenesis for over 20 years. Strain CNCTC10/84 has an unusually strong hemolytic activity, but the genetic basis is unknown. In this study, we discovered that a single-nucleotide insertion in an intergenic homopolymeric tract is responsible for the elevated hemolytic activity of CNCTC10/84.

**KEYWORDS** CNCTC10/84, CovR-CovS two-component regulatory system, *Streptococcus agalactiae*, homopolymeric tract, hyperhemolytic phenotype, single-nucleotide insertion

*Streptococcus agalactiae* (group B streptococcus [GBS]) is a leading cause of neonatal sepsis, pneumonia, and meningitis (1–3). It also causes invasive infections in pregnant women and immunocompromised patients (1, 4–11). GBS strains are commonly epidemiologically classified into one of 10 known serotypes (Ia, Ib, and II to IX) based on the antigenicity of capsular polysaccharide variants (12–14). Among these variants, serotypes Ia, Ib, II, III, and V cause the majority of infant invasive GBS infections (15).

GBS strain CNCTC10/84 is a capsule serotype V strain that was isolated from the blood of a septic neonate (16). It is highly virulent in animal models and has been used extensively in many laboratories for molecular pathogenesis investigations (17–33). CNCTC10/84 has strong hemolytic activity due to the overproduction of β-hemolysin, a key virulence factor of GBS (16, 21, 34–41). The β-hemolysin made by GBS is a

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pigmented cytotoxic lipid synthesized and transported by proteins encoded by the *cyl* operon (*cylX-cylK*) (29, 41–44). Multiple studies have demonstrated that strain CNCTC10/84 is hyperhemolytic and highly pigmented due to β-hemolysin overproduction (16, 45, 46). However, the genetic basis underlying the overproduction of β-hemolysin by CNCTC10/84 is unknown.

In GBS, the CovR-CovS (control of virulence) two-component system is a major global regulator that controls the expression of many virulence factors and metabolic enzymes (47). CovR-CovS regulation suppresses GBS production of β-hemolysin and, conversely, promotes that of CAMP factor (47). Typically, as a consequence, GBS strains that are functionally deficient in CovR-CovS regulation have enhanced beta-hemolytic activity and diminished CAMP factor activity (47–49). Given that CNCTC10/84 is phenotypically similar to a CovR-CovS-deficient strain (i.e., hyperhemolytic and with hypo-CAMP activity) (16, 50), we hypothesized that CNCTC10/84 would likely have a loss of function (frequently a reading frameshift) mutation in either the *covR* or *covS* gene. However, a genome sequencing study done by Hooven et al. found that CNCTC10/84 has intact *covR* and *covS* genes (50), and therefore factors other than *covR* or *covS* gene disruption are responsible for the hyperhemolytic phenotype of CNCTC10/84.

In this study, we demonstrate that a single-nucleotide insertion in the *covR* promoter region is responsible for the unusual hyperhemolytic and hypo-CAMP factor activities of CNCTC10/84. We also show that this insertion is beneficial for GBS survival in human whole blood *ex vivo*.

## RESULTS

**A single-nucleotide insertion in the *covRS* promoter confers altered hemolytic activity and CAMP factor activity on GBS strain CNCTC10/84.** Given that strain CNCTC10/84 is phenotypically like GBS strains lacking CovR-CovS regulation but has intact *covR-covS* genes, we inspected the *covRS* upstream untranslated region for polymorphisms that potentially affect promoter function. Compared to GBS strains with the wild-type allele of the *covRS* promoter (such as serotype 1a strain A909), CNCTC10/84 has a single-nucleotide insertion in a homopolymeric nucleotide tract in the *covRS* promoter region (Fig. 1A). The wild-type *covRS* promoter has 9 consecutive "Ts" downstream of the CovR binding motif and upstream of the *covR* start codon (48), while the CNCTC10/84 *covR* promoter allele has 10 Ts (Fig. 1A). Because the CovR-CovS two-component system directly regulates its own expression along with that of β-hemolysin and CAMP factor (47), we hypothesized that this single-nucleotide insertion alters *covR* and *covS* expression and consequently causes aberrant regulation and production of β-hemolysin and CAMP factor in CNCTC10/84. To test this hypothesis, we generated an isogenic mutant strain of CNCTC10/84 (i.e., CNCTC10/84-9T) with 9 Ts in the homopolymeric tract (Fig. 1A). An isogenic *covR-covS* knockout strain (i.e., CNCTC10/84-Δ*covRS*) was also generated for use as a CovR-CovS regulation-lacking control (Fig. 1). Our results show that both CNCTC10/84 and CNCTC10/84-Δ*covRS* were strongly hemolytic on sheep blood agar and highly pigmented on Todd-Hewitt broth supplemented with yeast extract (THY) agar (Fig. 1B). In contrast, both CNCTC10/84-9T and A909 with the wild-type promoter (9 Ts), were weakly hemolytic and produced little pigment (Fig. 1B). Quantitative assay of hemolytic activity showed that strains CNCTC10/84 and CNCTC10/84-Δ*covRS* caused significantly more hemolysis than strains CNCTC10/84-9T and A909 with the wild-type *covR* promoter (Fig. 1C). We also found that CNCTC10/84 and CNCTC10/84-Δ*covRS* had diminished CAMP activity, while strains CNCTC10/84-9T and A909 with the wild-type *covR* promoter had robust CAMP activity (Fig. 1D). In summary, we demonstrate that deleting a single nucleotide from the poly(T) tract of the CNCTC10/84 *covR* promoter region restores what appears to be near-wild-type levels of hemolysin and CAMP activity.

**A single-nucleotide insertion in the *covR* promoter significantly alters the expression of CovR-CovS-regulated genes in GBS strain CNCTC10/84.** We examined the effects of the *covRS* promoter region single-nucleotide insertion on CovR-CovS-

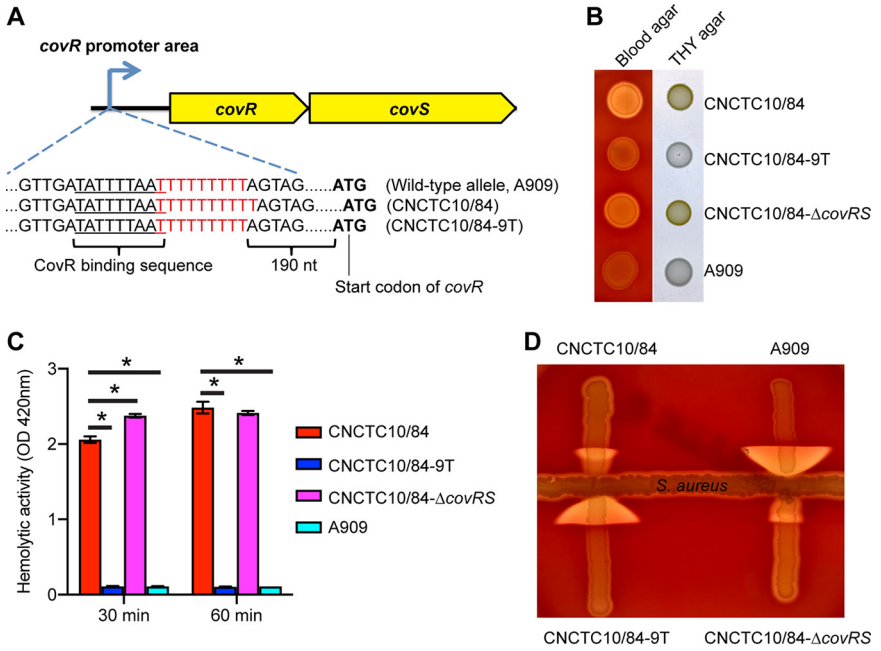


**FIG 1** A single-nucleotide insertion in the *covR* promoter confers altered hemolytic activity and CAMP activity on CNCTC10/84. (A) Schematic depiction of the *covR* upstream sequences of assayed strains. CovR binding sequence is underlined. (B) Hemolytic and pigmentation phenotypes of GBS strains. (C and D) Hemolytic activity (C) and CAMP activity (D) of GBS strains. OD, optical density. *, $P < 0.05$ versus 10/84 ($n = 4$, one-way analysis of variance with Dunnett's multiple-comparison test).

regulated gene expression. Relative transcription was determined using quantitative reverse transcription PCR (qRT-PCR) for strains cultured in Todd-Hewitt broth supplemented with yeast extract (THY). CNCTC10/84 with the native 10-T *covRS* promoter allele had very low *covR* and *covS* transcript levels (Fig. 2A). The CNCTC10/84-9T isogenic mutant with one T deleted from the homopolymeric tract had significantly increased transcript levels of both *covR* and *covS* (Fig. 2A). Not surprisingly, A909 with the wild-type *covR* promoter (9 Ts) had significantly higher levels of *covR* and *covS* transcripts than those of CNCTC10/84. As expected, *covR* and *covS* expression was undetectable in the *covR-covS* deletion strain CNCTC10/84-Δ*covRS* (Fig. 2A). These results show that the single-nucleotide insertion impairs the expression of *covR* and *covS* in CNCTC10/84.

We next examined the expression of β-hemolysin synthesis genes. The *cyl* operon that encodes genes required for the production of β-hemolysin is negatively regulated by the CovR-CovS two-component system (47). As expected, the *covR-covS* deletion strain CNCTC10/84-Δ*covRS* had high *cylE* and *cylI* transcript levels consistent with the lack of CovR-CovS repression (Fig. 2B). Similarly, CNCTC10/84 also had high *cylE* and *cylI* transcript levels (Fig. 2B), again consistent with a loss of CovR-CovS repression. In contrast, the isogenic mutant strain CNCTC10/84-9T had significantly lower *cylE* and *cylI* relative transcript levels (Fig. 2B, 10/84-ΔT), consistent with the single T deletion in the *covR* promoter restoring normal (wild-type-like) CovR-CovS repression/regulation. Strain A909 with the wild-type *covR* promoter had low transcript levels of the *cyl* genes, similar to those of CNCTC10/84-9T (Fig. 2B).

Inversely to the repression of the β-hemolysin synthesis genes, the CAMP factor-encoding *cfb* gene is positively regulated by CovR and CovS (47, 49). Consistent with the CAMP factor activity results (Fig. 1D), *cfb* expression was high in strains CNCTC10/84-9T and A909 with a wild-type 9-T *covR* promoter and normal *covR-covS* transcript levels but low in CNCTC10/84 and CNCTC10/84-Δ*covRS* with significantly lower *covR-covS* transcript levels (Fig. 2B).

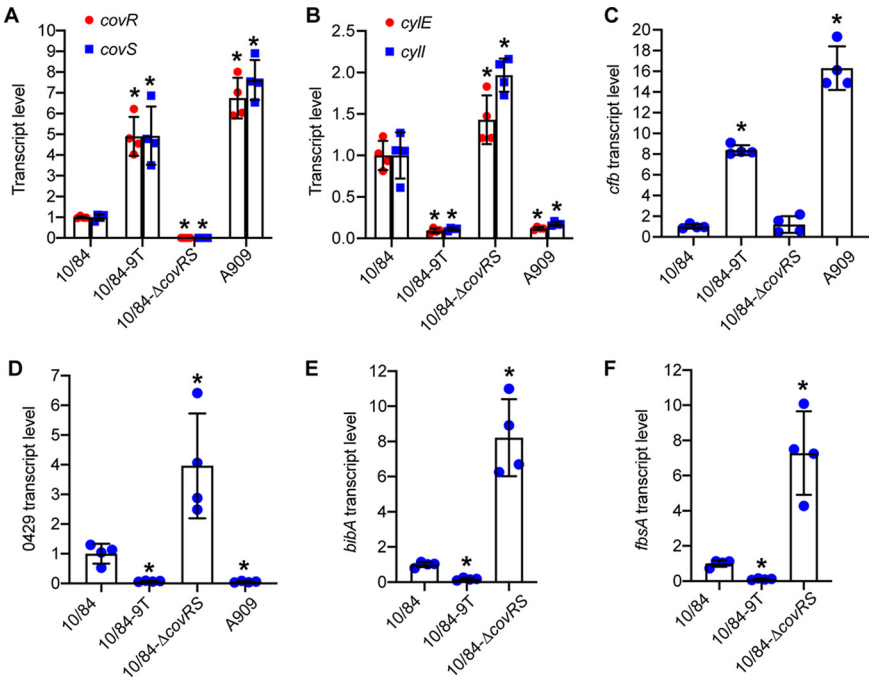


**FIG 2** A single-nucleotide insertion in the *covR* promoter confers altered expression of CovR-CovS-regulated genes in CNCTC10/84. Data are expressed as means ± standard deviation (SD). *, $P < 0.05$ versus 10/84 (one-way analysis of variance with Dunnett's multiple-comparison test). (E and F) A909 was not included because for *bibA* and *fbsA*, TaqMan primers and probes designed for strain CNCTC10/84 are not compatible with strain A909 due to sequence dissimilarity. (D) Locus number *0429* is relative to the genome of CNCTC10/84.

Lastly, to further investigate the effects of the single-nucleotide difference in the *covR* promoter poly(T) tract on GBS gene expression, we examined the three additional genes negatively regulated by CovR-CovS (47). These genes are *0429*, encoding a putative secreted protein, *bibA*, encoding a cell wall-anchored adhesin (51), and *fbsA*, encoding a fibrinogen-binding protein (52). We found that compared to CNCTC10/84, strain CNCTC10/84-9T had significantly lower expression of these three genes (Fig. 2D to F). Consistent with these genes being under CovR-CovS system repression, the *covR-covS* deletion strain CNCTC10/84-Δ*covRS* had relative transcript levels of these three genes that were significantly higher than those in strain CNCTC10/84 (Fig. 2D to F).

In summary, our gene expression data show that the single-nucleotide insertion in the *covR* promoter resulted in significantly decreased expression of *covR* and *covS*, as well as derepression of all CovR-CovS negatively regulated virulence factors examined.

**A single-nucleotide insertion in *covR* promoter confers increased survival of CNCTC10/84 in human whole blood.** Because CNCTC10/84 was isolated from the blood of a septic neonate, we examined the effect of the single-nucleotide insertion on GBS growth in human whole blood collected from two healthy donors (Fig. 3). Our results show that the strongly hemolytic strains CNCTC10/84 and CNCTC10/84-Δ*covRS* grew significantly better than the weakly hemolytic strain CNCTC10/84-9T in whole blood obtained from both donors (Fig. 3A and B). GBS causes hemolysis when interacting with human whole blood. After a 6-h incubation in the whole blood, strains CNCTC10/84 and CNCTC10/84-Δ*covRS* caused significantly more hemolysis than strain CNCTC10/84-9T (Fig. 3C and D). Moreover, the extent of hemolysis caused by the *covR-covS* knockout mutant was significantly higher than that for CNCTC10/84 (Fig. 3C and D). None of the GBS strains grew significantly differently in THY (Fig. 3D), suggesting that the differences in hemolysis observed in human whole blood are likely not attributable to basic differences in capacity of the strains to grow in a nutrient-rich

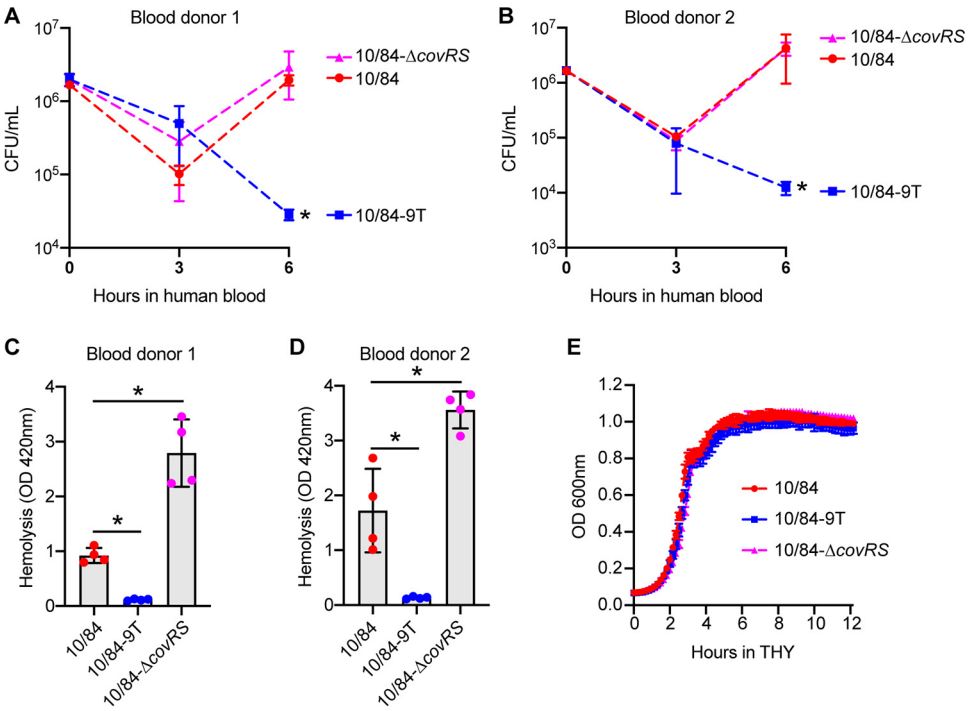


**FIG 3** A single-nucleotide insertion in the *covR* promoter contributes to CNCTC10/84 survival in human whole blood. (A and B) Growth of GBS strains in human whole blood. (C and D) Hemolysis caused by GBS strains after a 6-h incubation in human whole blood. (E) Growth of GBS strains in THY. (A to D) *, $P < 0.05$ versus 10/84 ($n = 4$, one-way analysis of variance with Dunnett's multiple-comparison test).

environment. In summary, our results show that the single-nucleotide insertion in the *covR* promoter confers significantly increased GBS survival in human whole blood *ex vivo*. Also, GBS strains with this single-nucleotide insertion caused significantly higher hemolysis when incubated with human whole blood.

**Inserting a single T into the homopolymeric tract of the wild-type *covR* promoter is sufficient to confer hyperhemolysis.** We showed that deleting a single T from the CNCTC10/84 *covR* promoter abolished the hyperhemolytic phenotype of this strain (Fig. 1). To examine if this is a strain-specific phenomenon unique to CNCTC10/84 or a strain-independent mechanism that has evolved in GBS for altering virulence factor expression by changing *covRS* expression, we constructed isogenic mutant strain A909-10T by adding a T to the *covR* promoter of strain A909, a GBS isolate with the wild-type allele of the *covR* promoter (9 Ts) (Fig. 4A). Our results showed that compared to the wild-type parental strain A909, the isogenic mutant strain A909-10T had significantly decreased expression of *covR*, significantly increased expression of the hemolysin gene *cylE*, and significantly decreased expression of the CAMP factor gene *cfb* (Fig. 4B to D). Consistent with the gene expression profile, isogenic mutant strain A909-10T had markedly increased beta-hemolysis and pigment production, yet decreased CAMP factor activity, similarly to CNCTC10/84 (Fig. 4E and F). In summary, our results show that adding a T to the wild-type *covR* promoter is sufficient to confer hyperhemolysis in A909.

***covR* promoter region polymorphisms in a large collection of GBS clinical isolates causing invasive infections.** To study if the CNCTC10/84-like insertion in the *covR* promoter region is present in GBS clinical isolates, we investigated the extent of natural variation in the homopolymeric T-nucleotide tract in the *covR* promoter among 6,516 GBS invasive infection isolates using kmer counting as previously described for a homopolymeric nucleotide tract in *Streptococcus pyogenes* (53). The 6,516 isolates were collected and whole-genome sequenced as part of the Active Bacterial Core surveillance program of the Emerging Infections Program of the Centers for Disease Control

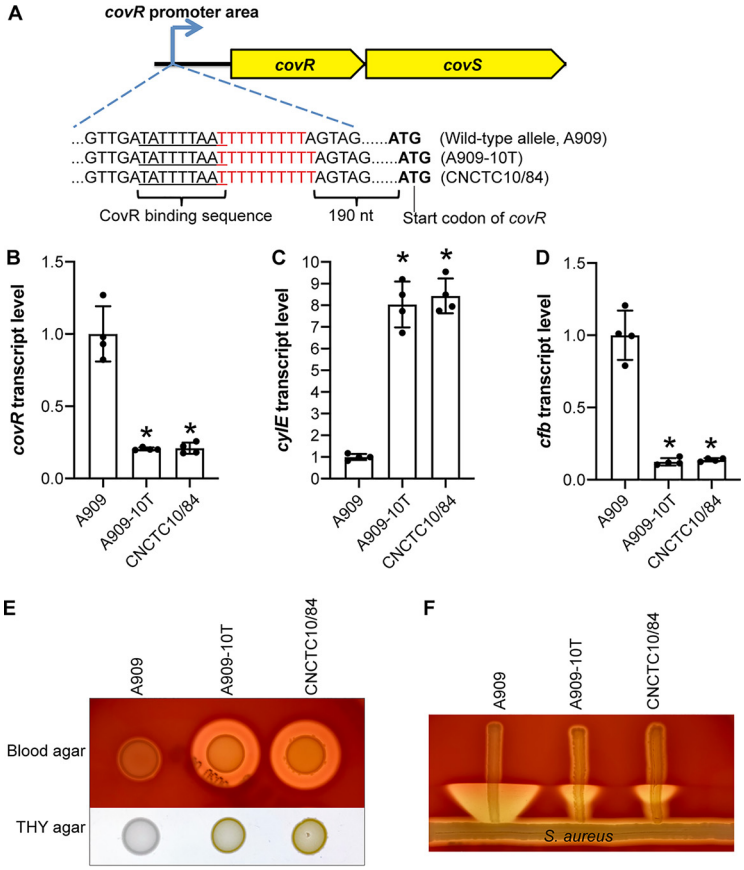


**FIG 4** Inserting a single T into the wild-type *covR* promoter of strain A909 is sufficient to confer hyperhemolysis. (A) Schematic depiction of the *covR* upstream sequences of assayed strains. (B to D) Transcript levels of *covR*, the hemolysin gene *cylE*, and the CAMP factor gene *cfb*. *, $P < 0.05$ versus A909 ($n = 4$, one-way analysis of variance with Dunnett's multiple-comparison test). (E) Hemolysis and pigment phenotype of GBS strains. (F) CAMP factor activity of GBS strains.

and Prevention (54) (BioProject accession number PRJNA355303). The number of sequencing reads matching from 6 Ts to 13 Ts was determined for 6,509 of the 6,516 isolates (Fig. 4). Nearly all of the *covRS* promoters, 6,495 (99.8%), were wild type/A909 like and had 9 Ts. Eleven isolates had 8 Ts, and only 3 isolates were CNCTC10/84 like and had 10 Ts. None of the isolates were found to have 6, 7, 11, 12, or 13 Ts. Thus, the *covRS* promoter T-nucleotide tract was nearly invariant among the GBS invasive infection isolates examined.

## DISCUSSION

In this study, we discovered the genetic basis underlying the hyperhemolysis of GBS strain CNCTC10/84, a highly virulent strain that has been used extensively to study GBS pathogenesis. Our results show that a single-nucleotide insertion in the *covR* promoter area is responsible for the enhanced hemolytic activity of CNCTC10/84. This mutation is beneficial for GBS survival in human whole blood *ex vivo*.

The CovR-CovS two-component system is an important global regulator of virulence in GBS (47, 48). CovR positively autoregulates its own expression via binding to the *covR* promoter (Fig. 1) (48). In this study, we demonstrate that CNCTC10/84 carries a single-nucleotide insertion in the *covR* promoter that results in strongly reduced promoter activity (Fig. 2). This insertion increases the length of the homopolymeric tract; however, it does not alter the *covR* binding motif (48) (Fig. 1A). We speculate that the insertion does not affect CovR binding to the CNCTC10/84 *covR* promoter but instead affects interaction between CovR and RNA polymerase. Proper interaction between CovR and the RNA polymerase complex is essential for *covR* expression (55).

In bacterial pathogens, it is not uncommon for intergenic homopolymeric tracts to play a role in gene regulation and virulence (56). For example, small insertions or deletions within an intergenic homopolymeric tract result in fimbrial phase variation in *Bordetella pertussis* (57). Similarly, length variation in a homopolymeric tract in the promoter region of *nspA* has been demonstrated to affect factor H-mediated serum resistance in *Neisseria meningitidis* (58). We recently discovered that a one-nucleotide indel in an intergenic homopolymeric tract significantly alters global transcript profiles and virulence of *Streptococcus pyogenes* (53, 59). In this study, we provide another example of intergenic homopolymeric tract polymorphism that affects promoter activity, specifically by altering hemolysin production and potentially virulence of GBS. Further study is needed to unravel the exact biological function of the homopolymeric tract in the *covR* promoter area.

Many studies have found that CNCTC10/84 is phenotypically similar to a *covR-covS*-deficient strain, due to its strong hemolytic activity and weak CAMP activity (16, 50, 60, 61). Here, we showed that CNCTC10/84 is a weak expresser of *covR* and *covS*, although it has intact *covR* and *covS* genes. We also show that CNCTC10/84 is phenotypically similar but not identical to an isogenic *covR-covS* knockout mutant strain (Fig. 2). For example, the expression of CovR-suppressed genes (*cylE*, *cylI*, *0429*, *bibA*, and *fbsA*) is significantly higher in the *covR-covS* knockout strain than in CNCTC10/84 (Fig. 2). Also, the *covR-covS* knockout strain had significantly higher hemolytic activity than CNCTC10/84 (Fig. 1 and 3). These results suggest that the derepression of CovR-suppressed genes is incomplete in CNCTC10/84, presumably due to the residual expression of *covR* and *covS* (Fig. 2A). Finally, our results show that there are slight differences in the *covR*- and *covS*-regulated genes between A909 and isogenic mutant CNCTC10/84-9T (Fig. 2A and C). We speculate that the moderate difference in gene expression profiles is due to the distinct genetic backgrounds of A909 and CNCTC10/84.

In this study, we demonstrate that the single-nucleotide insertion in the *covR* promoter is beneficial for CNCTC10/84 survival in human whole blood (Fig. 3). Changing this promoter to the wild-type allele significantly impaired GBS growth in whole blood (Fig. 3). We speculate that CNCTC10/84 is more resistant to the neutrophils present in the whole blood due to the overproduction of the cytotoxic $\beta$-hemolysin. In support of this hypothesis, Liu et al. showed that $\beta$-hemolysin is essential for GBS survival in human whole blood and for resisting neutrophil killing by promoting cell death (34). We also showed that CNCTC10/84 overexpresses the virulence genes *bibA* and *fbsA* (Fig. 2), which encode surface-located adhesins known to protect GBS from opsonophagocytosis and which are required for GBS survival in human whole blood (51, 52). Therefore, the genetic bases underlying enhanced survival of CNCTC10/84 in whole blood could be multifactorial.

Although the insertion in the *covRS* promoter is beneficial for CNCTC10/84 growth in whole blood *ex vivo* (Fig. 3), we found that the mutation is rare among clinical isolates causing invasive infections. The large majority of the clinical isolates have the wild-type *covR* promoter. This indicates that proper expression of *covR* and *covS* is critical for GBS fitness and pathogenesis during human infections. That is, decreased expression or complete loss of CovR and CovS function may result in a fitness loss under certain intrahost conditions. Prior studies demonstrate that deletion of *covR* and *covS* in GBS can result in increased or decreased pathogenesis depending on the infection models used. For example, Lembo et al., showed that infection with CovR-deficient GBS strains resulted in increased sepsis in mice when injected intravenously (48). Also, CovR-deficient GBS strains were more proficient in induction of permeability and proinflammatory signaling pathways in brain endothelium and penetration of the blood-brain barrier (48). Conversely, there is compelling evidence showing that CovRS-deficient GBS strains are less fit in other infection models. For instance, a *covR-covS* knockout mutant was significantly impaired for persistence in human serum (47). Also, loss of CovS/CovR abrogates intracellular survival of a type III GBS in macrophages (62). Moreover, CovR-deficient GBS strains exhibit a decreased ability to invade brain micro-

vascular endothelial cells and lung epithelial cells (48). Furthermore, a CovR-deficient serotype III GBS strain was significantly attenuated for colonization in mice and adhesion to uroepithelial cells (63). Lastly, CovR and CovS mutant strains were significantly attenuated for causing sepsis in mice when injected intraperitoneally (49). It is noteworthy that for intraperitoneal injection, GBS cells must invade the epithelium to gain access into the bloodstream and cause sepsis, and CovRS-deficient GBS strains have a defect in crossing epithelial barriers. Collectively, these results suggest that the CovRS two-component system is critical for colonization and epithelial cell invasion. Although CovRS-deficient strains (decreased *covR-covS* expression, or loss of CovRS function) may have advantages in the bloodstream due to the enhanced production of cytotoxin, these strains may have defects in transmission, colonization, and crossing epithelial barriers. Nevertheless, it is interesting that the GBS *covR* promoter has a homopolymeric tract that regulates *covR-covS* expression. The presence of this homopolymeric tract may favor GBS transition from a commensal to an invasive bacterial pathogen. Further investigations are needed to test this hypothesis.

## MATERIALS AND METHODS

**Construction of isogenic mutant strains.** All of the mutant strains used in this study were constructed using allelic exchange as described previously (64). Isogenic mutant strain CNCTC10/84-9T was derived from CNCTC10/84 by deleting one T in the homopolymeric tract in the *covR* promoter region. CNCTC10/84-Δ*covRS* was constructed by deleting the *covR* and *covS* genes of CNCTC10/84. Isogenic mutant strain A909-10T was derived from A909 by inserting a T into the homopolymeric tract of the *covR* promoter. Primers used for mutant construction are listed in Table S1 in the supplemental material. All isogenic mutant strains were whole-genome sequenced as the final confirmation of the allelic replacement and to rule out the introduction of unwanted spurious mutations influencing *covR-covS* expression and GBS hemolytic activity. Briefly, the genomes of GBS strains were sequenced using an Illumina NextSeq 550 instrument. Sequence reads were quality filtered, adapter and artifact trimmed, and base call error corrected using Trimmomatic and Musket (65) and then mapped to the genome of CNCTC10/84 or A909 using SMALT (https://www.sanger.ac.uk/tool/smalt-0/). Single-nucleotide polymorphisms (SNPs) and insertions and deletions (indels) were identified using FreeBayes and Pilon (66).

**Quantification of hemolytic activities of GBS strains.** A hemolytic activity assay was performed as described previously (16), with minor modifications. Briefly, GBS cells ($10^8$ CFU) were inoculated into 10 ml of sheep red blood cells (1%) suspended in phosphate-buffered saline (PBS) supplemented with 0.2% glucose and then incubated at 37°C. After an indicated period of incubation, the tubes were centrifuged to pellet intact red blood cells and GBS cells. An aliquot of 100 $\mu$l of the supernatant was transferred to a 96-well plate. Hemolysis was assessed by measuring hemoglobin released into the supernatant by absorbance at 420 nm.

**Quantitative reverse transcription-PCR analysis of gene expression in GBS strains.** GBS strains were grown in THY broth (Todd-Hewitt broth supplemented with yeast extract) to an optical density (OD) at 600 nm of 0.5. RNA from GBS cultures was extracted with an RNeasy minikit (Qiagen) and converted into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR was performed with the TaqMan Fast Universal PCR master mix (Applied Biosystems) and an ABI 7500 Fast System (Life Technologies) instrument. The sequences of the TaqMan primers and probes for the assayed genes are listed in Table S2 in the supplemental material. Each experiment was performed in quadruplicate (four biological replicates). A no-template control (NTC) was included to rule out false-positive signals generated by contamination or primer dimer formation. The statistical significance of relative expression differences between strains was evaluated using one-way analysis of variance with Dunnett's multiple-comparison test.

**GBS survival in human whole blood.** Heparinized human blood was collected from healthy volunteers under a Houston Methodist Research Institute Institutional Review Board human subject protocol (protocol number Pro00004933) and processed as described previously (67). To compare the ability of GBS strains to grow in human whole blood, GBS strains were grown to the mid-exponential phase in THY broth (OD of 0.5). GBS cells were washed three times with 10 ml of PBS and suspended with an equivalent volume of PBS. An aliquot of 20 $\mu$l of the GBS cell suspension (approximately $2 \times 10^6$ CFU) was inoculated into 2 ml of human whole blood and incubated at 37°C in 2-ml tubes rotated horizontally side over side. Aliquots (100 $\mu$l) of the seeded blood samples were recovered at 0, 3, and 6 h postinoculation, and GBS CFU were enumerated by serial dilution and growth on blood agar plates.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, XLSX file, 0.04 MB.

**SUPPLEMENTAL FILE 2**, XLSX file, 0.03 MB.

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