Reevaluation of whether a Functional Agr-like Quorum-Sensing System Is Necessary for Production of Wild-Type Levels of Epsilon-Toxin by *Clostridium perfringens* Type D Strains

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**ABSTRACT** *Clostridium perfringens* type B and D strains produce epsilon-toxin (ETX). Our 2011 *mBio* study ([mBio](https://doi.org/10.1128/mBio.00275-11), 2011, [5]) reported that the Agr quorum-sensing (QS) system regulates ETX production by type D strain CN3718. However, subsequent studies have brought that conclusion into question. For example, we reported in 2012 ([Infect Immun](https://doi.org/10.1128/IAI.00438-12), 2012, [6]) that the Agr-like QS system is not required for wild-type ETX production levels by two type B strains. Consequently, we reexamined whether the Agr-like QS system regulates ETX production in type D strains by using Targetron insertional mutagenesis to construct new *agrB* null mutants of two type D strains, CN3718 and CN2068. Western blotting showed that both *agrB* mutants still produce wild-type ETX levels. However, the newly constructed *agrB* mutants of both type D strains produced reduced amounts of alpha-toxin, and this effect was reversible by complementation, which confirms loss of functional AgrB production by these mutants since alpha-toxin production is known to be regulated by AgrB. Coupled with the previously published results for type B strains, these new findings indicate the Agr-like QS system is not usually necessary for *C. perfringens* to produce wild-type ETX levels.

**IMPORTANCE** Since epsilon-toxin (ETX) is necessary for the virulence of *C. perfringens* type D and, likely, type B strains, understanding the regulation of ETX production is important. In 2011, we reported that an *agrB* null mutant of type D strain CN3718 produces less ETX than its wild-type parent. However, when new *agrB* mutants were constructed in type D strains CN3718 and C2068, ETX production was unaffected. Those newly constructed *agrB* mutants produced less alpha-toxin, and this phenotype was reversible by complementation, confirming construction of *agrB* null mutants since alpha-toxin production is regulated by AgrB. Coupled with previous results for type B strains, these new type D results support the conclusion that the Agr QS is not usually necessary for wild-type ETX production levels.
two-component regulatory system (TCRS) is required for production of wild-type ETX levels by two type B strains (6), (ii) production of all other C. perfringens toxins regulated by the Agr QS system also involves the VirS VirR TCRS (7–14), and (iii) the Agr QS signal peptide can bind directly to VirS as a receptor (15).

Due to those apparent discrepancies, we recently constructed a second agrB mutant and complementing strain in a different stock culture of CN3718 than that used in 2011 (Fig. 1A). This new agrB mutant was constructed by Targetron-mediated insertion of an ~900-bp product into their agrB gene. DNA from each strain was digested with EcoRI and electrophoresed on a 1% agarose gel prior to blotting and hybridization with the intron-specific probe. (C) RT-PCR evaluation of agrB expression shows that the agrB mutants (left, CN3718 agrB KO; right, CN2068 agrB KO) expressed an intron::agrB fusion transcript, while the complementing strains (CN3718 agrB Comp and CN2068 agrB Comp) expressed the wild-type agrB transcript. These PCR assays were repeated three times, and a representative result is shown. For size reference, a 1-kb marker is shown (Fisher Scientific).
To confirm that introduction of an intron into the agrB gene in CN3718 had created an agrB null mutant, alpha-toxin (CPA) production by these cultures of CN3718 or its derivatives was also evaluated, since CPA production in C. perfringens is regulated by AgrB (12, 13). Consistent with the expected phenotype of an agrB null mutant, less CPA was produced by this mutant versus its parent, and this effect was reversible by complementation (Fig. 2C).

To further evaluate whether the Agr QS is necessary for production of wild-type ETX levels by type D strains, an agrB mutant and complementing strain were similarly constructed in CN2068 (18), a second type D strain (Fig. 1A). Southern blot analysis (Fig. 1B) demonstrated that the CN2068 agrB mutant contained only a single intron insertion. RT-PCR (Fig. 1C) showed that this CN2068 agrB mutant expressed an intron:agrB fusion transcript and that complementation had restored expression of the wild-type agrB transcript. Under the same culture conditions used in our 2011 mBio paper (5), no growth differences were noted between CN2068 and its agrB mutant (data not shown). Western blotting confirmed that, under these culture conditions, CN2068 produced ETX, although in smaller amounts than CN3718 (Fig. 2A). Western blotting also detected no differences in ETX production between the CN2068 agrB mutant and its wild-type parent or the complementing strain. In contrast, Western blots of the same cultures showed that this agrB null mutant produced much less CPA than wild-type CN2068 and that complementation substantially restored production of this toxin (Fig. 2C).

Coupling the new results presented above with our previous results indicating that inactivating the Agr QS does not affect ETX production levels by two type B strains (6), we conclude that the Agr QS is not usually necessary for type B or D strains to produce wild-type levels of ETX.

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