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Analysis of the virulence-associated RevSR two-component signal transduction system of *Clostridium perfringens*

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ABSTRACT

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming bacterium that causes human gas gangrene (clostridial myonecrosis) and food poisoning. Early studies showed that virulence was regulated by the VirSR two-component signal transduction system. However, our identification of the RevR orphan response regulator indicated that more than one system was involved in controlling virulence. To further characterize this virulence-associated regulator, gel mobility shift experiments, coupled with DNase I footprinting, were used to identify the RevR DNA binding sequence. Bioinformatics analysis suggested that an orphan sensor histidine kinase, CPE1757 (renamed RevS), was the cognate sensor of RevR. Interaction between RevS and RevR was demonstrated by use of a bacterial two-hybrid system and validated by protein-protein interaction studies using biolayer interferometry. To assess the involvement of RevS in virulence regulation, the *revS* gene was inactivated by Targetron insertion. When isogenic wild-type, *revS* and complemented *revS* strains were tested in a mouse myonecrosis model, the *revS* mutant was found to be attenuated in virulence, which was similar to the attenuation observed previously with the *revR* mutant. However, transcriptional analysis of selected RevR-regulated genes in the *revS* mutant revealed a different pattern of expression to a *revR* mutant, suggesting that the RevSR system is more complex than originally thought. Taken together, the results have led to the identification and characterization of the two essential parts of a new regulatory network that is involved in the regulation of virulence in *C. perfringens*.

1. Introduction

Clostridium perfringens is a Gram-positive, spore-forming anaerobe that is found ubiquitously in the environment and is part of the normal flora of the human and animal gastrointestinal tract (Uzal et al., 2014). However, it is also a pathogen and has the ability to cause a wide range of histotoxic and enterotoxaemic diseases, which is attributed to the production of at least 20 different potent toxins and extracellular enzymes (Revitt-Mills et al., 2015; Uzal et al., 2014). The production of some of these toxins is regulated by the VirSR two-component signal transduction system (Cheung et al., 2010; Lyristis et al., 1994; Ma et al., 2011; Shimizu et al., 1994; Shimizu et al., 2002). In addition, a novel response regulator, RevR, has been shown to regulate the production of several potential virulence factors (Hiscox et al., 2011).

Many bacterial pathogens use two-component signal transduction systems to adapt to changes in their environment and to regulate virulence. Mutation of the genes encoding these systems often leads to virulence attenuation (Beier and Gross, 2006; Calva and Oropeza, 2006). In general, two-component systems consist of a membrane-bound sensor histidine kinase and its cytoplasmic cognate response regulator. Following the detection of a specific stimulus, the sensor histidine kinase autophosphorylates at a conserved histidine residue. The phosphoryl group then is transferred from the histidine to a conserved aspartate residue in the cognate response regulator. Once phosphorylated, the response regulator is able to control the expression of its target genes (Capra and Laub, 2012; Jung et al., 2012). The genes encoding the cognate pair are generally found adjacent to each other and often are co-transcribed (Capra and Laub, 2012). However, in some cases, the gene encoding one component is not accompanied by the gene of its cognate

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pair. The encoded proteins are referred to as orphans (Raghavan and Groisman, 2010).

RevR is an orphan response regulator that has sequence similarity to PhoB from *Clostridium kluyveri* and YycF from *Bacillus subtilis* (Hiscox et al., 2011). Bioinformatics analysis of the RevR amino acid sequence indicated that residues D11, E12 and K106 would form the phosphoacceptor pocket in the *N*-terminal region of the protein, while D54 was predicted to be the site of phosphorylation. A winged-helix DNA binding domain with structural similarity to similar domains in PhoB from *Escherichia coli* and YycF from *B. subtilis* was identified in the *C*-terminal region (Hiscox et al., 2011). Most importantly, RevR was shown to regulate virulence in a gas gangrene isolate, since mutation of the *revR* gene led to an attenuation in virulence (Hiscox et al., 2011).

The aim of this work was to further characterize RevR and its regulatory network. In this study we have shown that RevR recognizes and binds to PHO box-like sequences located upstream of the *pstS1* and *pstS2* phosphate uptake-related genes, and through protein-protein interaction assays and phosphotransfer experiments we have identified its cognate sensor histidine kinase, RevS. Virulence studies showed that RevS was also involved in regulation of virulence of *C. perfringens*. Taken together, these results delineate the new virulence-associated RevSR two component signal transduction system.

2. Materials and methods

2.1. Strains, plasmids, media and culture conditions

The bacterial strains and plasmids used in this work are shown in Table 1. *E. coli* strains were grown at 37 °C in 2x YT agar or broth or SOC broth (Sambrook et al., 1989) supplemented with chloramphenicol (30 μg/ml), ampicillin (100 μg/ml) or kanamycin (20 μg/ml). For bacterial two-hybrid analysis, 50 μl of 2% (w/v) 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-Gal) and 10 μl of 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) were spread on the surface of agar plates.

C. perfringens strains were cultured at 37 °C in tryptone-peptone-glucose (TPG) broth (Rood et al., 1978), brain heart infusion broth (Oxoid), fluid thioglycolate medium (FTG) (Difco), or nutrient agar supplemented with 30 μg/ml chloramphenicol ($NACm_{30}$), 10 μg/ml rifampicin and 10 μg/ml nalidixic acid ($NARif_{10}Nal_{10}$), or 7 μg/ml or 50 μg/ml erythromycin ($NAEm_7$ or $NAEm_{50}$, respectively). All agar cultures of *C. perfringens* were incubated in an atmosphere of 10% (v/v) H_2 and 10% (v/v) CO_2 in N_2.

2.2. Molecular techniques

Plasmid DNA from *E. coli* cells was routinely isolated using the QIAprep miniprep kit (Qiagen) as per the manufacturer's instructions or by an alkaline lysis method (Morelle, 1989). Restriction endonucleases and other enzymes were used as specified by the manufacturer (Roche, New England Biolabs).

PCR amplification was carried out as described previously (Cheung et al., 2004). PCR products were either extracted from agarose gels using the QIAquick® Gel Extraction Kit (Qiagen), or purified directly using the QIAquick® PCR Purification Kit (Qiagen). All PCR products were sequenced using the PRISM BigDye Terminator Mix (Applied Biosystems) to ensure that no errors had been incorporated during amplification. The oligonucleotide primers used in this study are listed in Table 2.

Chemically competent *E. coli* cells were made and transformed as before (Inoue et al., 1990). Transformation of *C. perfringens* cells was performed by electroporation (Scott and Rood, 1989) with 3–5 μg of purified plasmid DNA. *C. perfringens* chromosomal DNA was isolated as described previously (O'Connor et al., 2006).

2.3. Construction of the revS targetron plasmid

A group II intron was re-targeted to insert between positions 32/33 (relative to the ATG start codon) in the antisense DNA strand of *revS*. Primer-mediated mutation by PCR using the Targetron primers and the subsequent cloning of the re-targeted 349 bp fragment into the *Hin*dIII/*Bsr*GI site of pJIR3566 was carried out as described before (Cheung et al., 2010). The resultant *revS* Targetron plasmid, pJIR3883 (Table 1), was used in the construction of the *revS* mutant.

2.4. Construction of the revS complementation plasmid

The *revS* complementation plasmid was constructed as follows. The *revS* gene may be co-transcribed with the upstream gene, *cpe1758* (Fig. 1B). Therefore, to ensure that *revS* would be expressed from its native promoter, a 180 bp region containing a putative promoter upstream of *cpe1758* was PCR amplified using primers JRP5672 and JRP5673, which introduced *Asp*718 and *Bam*HI sites, respectively. The resultant product was digested and ligated into the corresponding sites of pJIR750, to give pJIR4222. At the same time, a 1.77 kb PCR product encoding the *revS* ribosome binding site and the *revS* gene was generated with JRP5674 and JRP5675. These primers incorporated *Bam*HI and *Pst*I sites at the 5′ and 3′ ends of the product, which facilitated its ligation into the corresponding sites in pJIR750, to give pJIR4227. Finally, this 1.77 kb fragment was subcloned into the *Bam*HI/*Pst*I site of pJIR4222, to give the *revS* complementation plasmid, pJIR4252.

2.5. Construction and complementation of the revR and revS mutants

The *revS* mutant, JIR12759, was constructed by Targetron insertion using pJIR3883, by the method described previously (Cheung et al., 2010). To ensure that all mutants were made from the same wild-type strain, at the same time a new *revR* mutant was constructed by allelic exchange using the suicide plasmid pJIR3429, as before (Hiscox et al., 2011). All mutants were confirmed by PCR analysis and Southern hybridization (data not shown). To complement the mutations, the *revR* and *revS* mutants were transformed with pJIR3534 (Hiscox et al., 2011) and pJIR4252, respectively.

2.6. Construction of the revR and revSc expression plasmids

To construct the expression plasmids, a 0.704 kb PCR product encoding the *revR* gene was amplified using primers JRP4267 and JRP4268 and a 1.158 kb PCR product carrying a truncated *revS* gene, *revSc,* was generated using JRP5849 and JRP5850. The truncation resulted in the production of the RevSc protein, which excluded the putative *N*-terminal transmembrane domains, but retained the cytoplasmic HAMP linker, PAS, HisKA and HATPase domains. In the PCR reactions, the forward primers (JRP4267, JRP5849) and reverse primers (JRP4268, JRP5850) incorporated *Nde*I and *Xho*I restriction sites at the 5′ and 3′ ends, respectively. These sites facilitated the insertion of the DNA fragment into the pET-22b(+) expression vector (Novagen) to construct the *revR* and *revSc* expression vectors, pJIR3721 and pJIR4361, respectively. Each expression construct was sequenced to confirm that the gene was in frame and free of mutations.