12-1-2016

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Recommended Citation

Garrett, S., Garrison-Schilling, K., Cooke, J., & Pettis, G. (2016). Capsular polysaccharide production and serum survival of Vibrio vulnificus are dependent on antitermination control by RfaH. *FEBS Letters, 590* (24), 4564-4572. https://doi.org/10.1002/1873-3468.12490

FEBS Letters

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Capsular polysaccharide production and serum survival of *Vibrio vulnificus* are dependent on antitermination control by RfaH

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(Received 22 September 2016, revised 6 November 2016, accepted 10 November 2016, available online 28 November 2016)

doi:10.1002/1873-3468.12490

Edited by Renee Tsolis

The human pathogen *Vibrio vulnificus* undergoes phase variation among colonial morphotypes, including a virulent opaque form which produces capsular polysaccharide (CPS) and a translucent phenotype that produces little or no CPS and is attenuated. Here, we found that a *V. vulnificus* mutant defective for RfaH antitermination control showed a diminished capacity to undergo phase variation and displayed significantly reduced distal gene expression within the Group I CPS operon. Moreover, the *rfaH* mutant produced negligible CPS and was highly sensitive to killing by normal human serum, results which indicate that RfaH is likely essential for virulence in this bacterium.

Keywords: capsular polysaccharide; phase variation; serum survival; transcriptional antiterminator

Vibrio vulnificus is a Gram-negative marine bacterium that colonizes the gut of oysters and poses a significant health risk to humans who consume raw or undercooked shellfish or who have wounds exposed to the bacterium. Ingestion of the bacterium can lead to a primary sepsis, which develops rapidly and with a substantial rate of mortality, while wound exposure can result in a severe necrotizing fasciitis. An important factor in disease progression is the ability of the bacterium to circumvent the host immune response. This avoidance is due in large part to the presence of capsular polysaccharide (CPS), which provides resistance to opsonization and phagocytosis, as well to the bactericidal action of complement in human serum; moreover, CPS production by *V. vulnificus* masks other immunogenic surface structures that could normally activate nonspecific immune responses [1,2].

Vibrio vulnificus undergoes reversible phase variation of exopolysaccharide production, including CPS, at high frequencies and in response to various environmental cues [3–5]. Phase variable colonial morphotypes include opaque, which produces CPS and is highly virulent in an iron-loaded mouse model, and translucent, which expresses little or no CPS and is greatly attenuated for virulence [2]. A third colonial phenotype is rugose, which expresses a separate exopolysaccharide and is virulent if it also produces CPS [6]. Multiple underlying genetic or epigenetic mechanisms appear to exist for phase variation in *V. vulnificus*. Beginning with an opaque parental strain, Chatzadaki-Livanis *et al.* [7] found that deletions of the *wzb* gene within the Group I CPS biosynthesis, transport, and assembly operon resulted in phase-locked translucent variants; meanwhile, no alterations within this operon were observed for translucent derivatives that could still reversibly switch back to opaque.

In a variety of Gram-negative bacteria, the production of surface components, including

Abbreviations
CPS, capsular polysaccharide; LB, Luria–Bertani; LPS, lipopolysaccharide; NHS, normal human serum.

exopolysaccharides, biofilm, and lipopolysaccharide (LPS), as well as other secreted virulence factors, is known to be regulated by the transcriptional antiterminator RfaH. The RfaH protein allows RNA polymerase to proceed past Rho-dependent termination sites without interrupting transcription, thereby improving processivity and preventing polarity within cognate operons [8,9]. An operon polarity suppressor (*ops*) is found upstream of the first gene within RfaH-controlled operons, and it is composed of a short highly conserved sequence, which functions by recruiting RfaH to the transcription elongation complex [8,9]. Mutations in the *rfaH* gene generally result in attenuation but the specific effects on virulence-related functions can vary. For example, while an *rfaH* mutant of uropathogenic *Escherichia coli* showed an altered LPS phenotype, produced less K15 capsule and alpha hemolysin, and was less resistant to human serum [10], a *Yersinia enterocolitica rfaH* mutant produced less LPS and was attenuated in its stress response, but was more resistant to serum killing [11].

An *ops* element was previously identified within the Group I CPS operon of *V. vulnificus*, which implies RfaH control of CPS production in this bacterium [12]; however, no characterization of RfaH regulation in *V. vulnificus*, or in any other *Vibrio* spp., has so far been performed. Here, we assessed the effects of insertion into the *rfaH* gene of *V. vulnificus* on CPS production and gene expression, as well as serum resistance. We found that the *rfaH* mutant appeared translucent on agar media and showed a reduced capacity to switch phases. Additionally, distal gene expression with the Group I CPS operon and CPS production were greatly reduced and serum resistance appeared to be abolished. As discussed later, our findings may have implications for recent efforts aimed at producing a stable vaccine strain for this pathogen.

Materials and methods

Bacterial strains and culture conditions

Vibrio vulnificus opaque clinical isolates YJO16 [13] and 1003(O) [14] have been described previously. *Vibrio vulnificus* strains were grown in heart infusion broth (Difco, Detroit, MI, USA) supplemented to 2% NaCl (HI broth) with 0.2% arabinose, and appropriate antibiotics. For growth on agar plates, 0.2% arabinose and 18 g of agar (Difco) per liter of HI were added along with necessary antibiotics. All broth cultures were incubated at 30 °C and shaken at 200 r.p.m.; plates were incubated overnight for 16–24 h at 30 °C. Phenotypic switching assays were performed as described previously using Luria–Bertani (LB) broth supplemented to 2% NaCl (LB2 broth) and 1 mM $CaCl_2{\cdot}2H_2O$ [3]. *Escherichia coli* strain BRL2288 [15], which was used for cloning, and strain S17.1 [16], which was used for intergeneric conjugations, have been described and were grown on LB plus appropriate antibiotics at 37 °C for 16–24 h.

Construction of an *rfaH* mutant

A 1160-bp chromosomal region containing the *rfaH* gene was amplified via PCR using primers RfaH-R (5′-TCTA GAGTCAGGTCGAGCAGTGAAAG-3′) and RfaH-F,2 (5′-CTGCAGGTACACCAATCCTGTGTAGG-3′) and purified YJO16 genomic DNA (gDNA), which was isolated as previously described [6]. Conditions for PCR were as detailed previously [17]. Following digestion with *Xba*I and *Pst*I, the resulting fragment was cloned into these same sites on plasmid pSP72 (Promega, Madison, WI, USA) to create pVV37. The insert region of pVV37 was confirmed by sequencing with relevant primers and BigDye v3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. An 840-bp nonpolar kanamycin-resistant cassette [18], which was obtained following digestion of plasmid pKan2 [17] with *Sma*I, was then inserted in the correct orientation at the *Xmn*I site of the cloned *rfaH* gene creating pVV38. This meant that the cassette was inserted following the 110th bp of the 507-bp *rfaH* gene. The 2-kb *Xba*I-*Pst*I fragment containing the cassette flanked by *rfaH* sequence was made blunted-ended using Klenow fragment; it was then cloned into the suicide vector pGP704sacB28 [19] at the plasmid's *Xba*I site, which had also been blunt-ended by using Klenow. The resulting plasmid, pVV39, was subsequently transformed into *E. coli* S17.1. Kanamycin-resistant, ampicillin-sensitive transconjugants, which resulted from double homologous recombination, were obtained from matings between *E. coli* S17 harboring pVV39 and *V. vulnificus* YJO16, which were performed as described previously [17]. Proper integration of the cassette was verified by PCR [6] using Amplitaq polymerase and primers RfaH-R, and RfaH-F,2. Southern blot hybridizations using radiolabeled probes for either the *rfaH* gene or the kanamycin resistance gene from plasmid pKan2 were performed as described previously [17] in order to confirm the *rfaH* mutant strain, which was designated YJ-01.

Complementation of YJ-01

A 703-bp region containing the *rfaH* gene was amplified via PCR using primers RfaH-F,3 (5′-CTGC AGTAGTCTGGCGAAATGCTAGG-3′) and RfaH-R (5′-TCTAGAGTCAGGTCGAGCAGTGAAAG-3′) and purified YJO16 gDNA. The *Pst*I- and *Xba*I-digested *rfaH* fragment was then cloned into these same sites on plasmid pSP72 to generate pVV59b. The insert region of the nascent plasmid was sequenced by using primers RfaH-F,3

and RfaH-R, and the method described in the previous section. The cloned *rfaH* gene was then released from pVV59b following digestion with *Pst*I and *Xba*I, and was inserted into these same sites behind the arabinose-inducible promoter on the chloramphenicol-resistant plasmid pBBRBAD2 [17] to create pVV60. Introduction of pVV60 or pBBRBAD2 into *V. vulnificus* YJ-01 was performed by intergeneric mating as described earlier.

Growth curves

Growth curves were performed according to Garrison-Schilling *et al.* [3] with minor modifications. Briefly, four independent colonies of each strain were inoculated into 3 mL of HI broth containing 0.2% arabinose and appropriate antibiotics, and these cultures were shaken overnight at 30 °C. Overnight cultures were diluted into 5 mL HI to an OD_{600} = 0.01 and incubated at 30 °C with shaking. OD_{600} readings were taken at 2, 4, 6, 9, and 24 h and each overnight culture was also spread on HI agar with 0.2% arabinose and appropriate antibiotics to confirm the absence of switching.

Reverse transcription quantitative real-time PCR

Total RNA was isolated by the method described by Grau *et al.* [6] from cultures grown to an OD_{600} = 0.4. Primers for reverse transcription quantitative real-time PCR (RT-qPCR) were tested by standard PCR and subsequent agarose gel electrophoresis using YJ016 gDNA. To generate cDNA, first strand synthesis was performed on 200 ng of clean RNA (A_{260}/A_{280} = 2.0) using Superscript® II (Invitrogen, Carlsbad, CA, USA) [6]. Primer efficiencies to determine appropriate primer and cDNA concentrations were conducted in duplicate using five sequential 1 : 10 dilutions of *V. vulnificus* YJ016 cDNA for the *wza* and *wbfY* genes, as well as the *tufA* reference gene, with controls that included water with reverse transcriptase (RT), gDNA without RT, and a nontemplate control (NTC) in a 96-well optical plate, which was run on the ViiA7 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using SYBR Select Master Mix chemistry (Applied Biosystems). Numerical efficiency was determined by the formula $E = 10^{(-1/\text{slope})} - 1$, and all calculations were made with the EXPRESSIONSUITE software v1.0.3 (Applied Biosystems). RT-qPCR was conducted on each sample versus each gene target in triplicate with 0.5 μL of appropriate 20 mM forward and reverse primers, 5 μL of cDNA, 12.5 μL SYBR Select Master Mix, and nuclease-free water to 25 μL. Samples were run alongside NTC, gDNA without RT, and water with RT controls on 96-well plates in the ViiA7 Real-Time PCR System. Gene expression was determined using the $\Delta\Delta C_t$ method for relative quantification within the accompanying EXPRESSIONSUITE software v1.0.3 (Life Technologies, Carlsbad, CA, USA). Each assay was repeated five times.

Human serum assay

Overnight HI cultures containing appropriate antibiotics were diluted 1 : 100 in 3 mL fresh HI media and grown to an OD_{600} = 0.45. Assays were based on the protocol previously described [20]. Cultures were spun down at 10 000 ***g***, washed once with phosphate-buffered saline (PBS), and then resuspended in 3 mL fresh PBS. Aliquots of approximately 4×10^7 CFU were removed, adjusted to 350 μL with PBS, and mixed with 650 μL of PBS, normal human serum (NHS) from AB plasma (Sigma, St. Louis, MO, USA), or killed NHS; the final reactions were then incubated at 37 °C for 1 h. Killed NHS was obtained by incubating NHS at 56 °C for 30 min and then cooling to room temperature. Additional aliquots of the original cell suspensions (again of approximately 4×10^7 CFU) were serially diluted, and duplicate 100-μL aliquots from the 10^{-5}, 10^{-6}, and 10^{-7} dilutions were plated on HI agar with relevant antibiotics in order to determine CFUs at time zero. After incubation, reactions were removed from the heat block and also plated in duplicate but at every serial dilution from 10^0 to 10^{-7}. Plates were incubated at 30 °C for 16–24 h prior to counting and the resulting CFU scores were then averaged for each strain. The assay was repeated at least three times for each strain.

Isolation and analysis of CPS

Capsular polysaccharide was isolated using the protocol by Lee *et al.* [21] with some modifications. Strains were grown overnight as 3-mL HI cultures containing appropriate antibiotics at 30 °C with shaking. Aliquots (100 μL) were spread on HI agar with appropriate antibiotics at 30 °C for 48 h. Bacterial lawns for each strain were aseptically removed from plates and suspended in 10 mL PBS at an OD_{600} = 100. Suspensions were incubated at 30 °C with shaking at 200 r.p.m. for 1 h and then centrifuged at 5000 ***g*** at 4 °C for 15 min. Pellets were washed with 0.45% NaCl, resuspended in 10 mL buffer (0.45% NaCl, 1% EDTA), and incubated at 4 °C for 1 h. Samples were then centrifuged at 10 000 ***g*** at 4 °C for 1 h and subsequently filtered with sterile 0.2 μm syringe filters. Filtered samples were treated with RNaseA, DNase I, and proteinase K, all as previously described [21]. Following extraction with phenol : chloroform (50 : 50) and chloroform, CPS was precipitated with 95% ethanol, washed with 70% ethanol, dried, and finally resuspended in distilled water. Equivalent volumes of the purified CPS extracts were then electrophoresed on 8% polyacrylamide stacking gels, and the polysaccharide was stained with Stains-All (SigmaAldrich.com) as described previously [22]. Two independent CPS isolations were performed for each strain. The galacturonic acid content in the CPS extracts was determined by using the colorimetric assay described previously [23]. The estimated carbohydrate concentration was expressed as ng of

galacturonic acid per μL of extract. From the two CPS isolations, a total of six galacturonic acid assays were performed with three replicates of each strain per assay.

Statistical analysis

ANOVA statistical analysis was conducted in Excel (Microsoft 2007) or by using SPSS software (IBM Corporation, Armonk, NY, USA). Statistical outputs were adjusted with Bonferroni's correction, a conservative method to control the familywise error rate in multiple comparisons. P values of < 0.05 were considered significant.

Results

An *rfaH* mutant of *V. vulnificus* shows a reduced propensity for phase variation

When a nonpolar kanamycin-resistant cassette was inserted into the *rfaH* gene (see Materials and methods for details) of opaque clinical isolate YJ016 (Fig. 1A), the resulting mutant, designated YJ-01, displayed a translucent phenotype on HI agar medium (Fig. 1B). Complementation using a cloned version of *rfaH* on the arabinose-inducible expression vector pBBRBAD2 (i.e., plasmid pVV60) restored opacity to YJ-01 (Fig. 1D), while, as expected, addition of the vector alone did not (Fig. 1C).

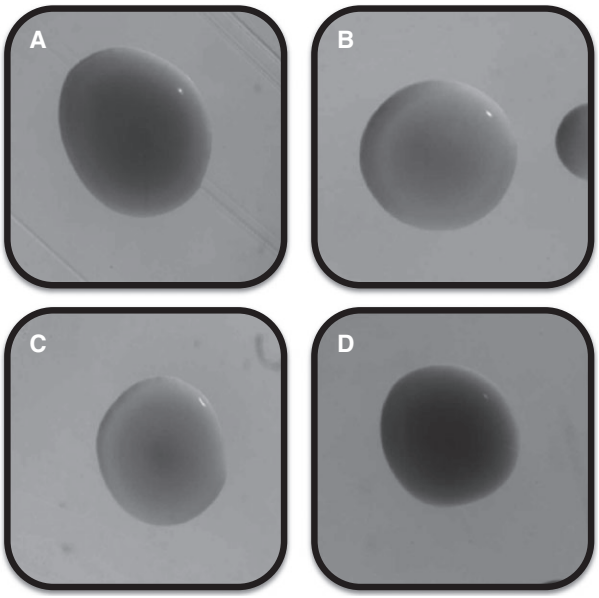

Fig. 1. Phenotype of the *rfaH* mutant YJ-01. *Vibrio vulnificus* strains were streaked on HI agar containing 0.2% arabinose (and kanamycin and chloramphenicol where appropriate) and incubated overnight at 30 °C. Panels: A, YJO16; B, YJ-01; C, YJ-01 (pBBRBAD2); D, YJ-01(pVV60).

The capacity of the *rfaH* mutant to undergo phenotypic switching was assessed by subjecting it to our previously describing switching assay, which includes daily passaging of strains in broth media with plating for individual colonies at regular intervals and subsequent counting and scoring of colonial phenotypes [3]. After 15 daily passages, 99.8% of the colonies of the translucent derivative YJ-01 remained translucent and no switching to either opaque or rugose had occurred (Fig. 2); the very few nontranslucent colonies seen for YJ-01 at 15 passages were of an indeterminant phenotype. This apparent lack of switching for YJ-01 was reminiscent of the phase-locked phenotype seen previously for translucent phase variants that had acquired deletions of the *wzb* gene, and it was in contrast to prior results for translucent variants that did not contain such CPS gene deletions and thus still underwent phase variation at readily detectable frequencies [7]. Meanwhile, switching here of the parental strain YJ016 was detected initially after 10 passages and, by 15 passages, $8.3 \pm 8.3\%$ of resulting colonies of YJ016 had switched from opaque to translucent, which was consistent with previous results for this strain [3]. The results for YJ016 were also similar to the complemented mutant YJ-01(pVV60) of which $17.3 \pm 9.9\%$ of its colonies had switched from opaque to translucent by 15 passages and $0.9 \pm 0.7\%$ had switched to rugose (Fig. 2). Growth curves for strain YJ-01 were nearly identical to those of the parent (Fig. S1), which indicated that the reduced capacity of the *rfaH* mutant to undergo phase variation was unlikely due to a putative indirect effect associated with slower growth.

RfaH controls CPS production in *V. vulnificus* at the transcriptional level

Typically, mutations in the *rfaH* gene lead to substantial decreases in distal gene transcription of RfaH-controlled operons, while proximal gene expression remains largely unaffected [8]. We examined the potential effects of *rfaH* inactivation on distal (*wbfY*) and proximal (*wza*) transcription of the Group I CPS operon [7] by isolating total RNA from mid-exponential cultures of YJ016, YJ-01, YJ-01 (pBBRBAD2), and YJ-01(pVV60), and quantifying transcription of these genes using RT-qPCR. As shown in Fig. 3, while there was little difference in expression of *wza* in the *rfaH* mutant YJ-01 compared to the parent YJ016, relative expression of *wbfY* was reduced over 25-fold (i.e., down to 0.03 ± 0.39) and this difference was considered significant based on an ANOVA analysis with adjustment by a Bonferroni *post hoc* test ($P < 0.001$). As expected, the addition of

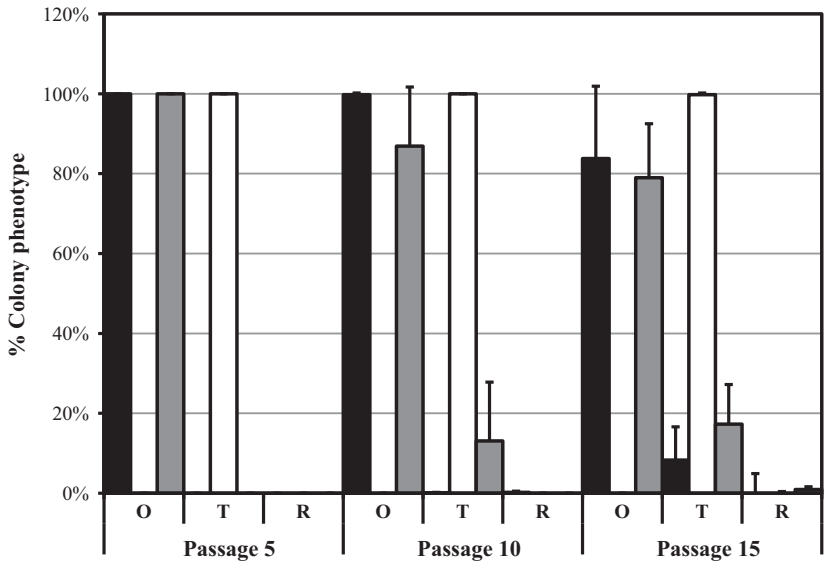

Fig. 2. Evidence of a reduced propensity for phase variation for the *rfaH* mutant. Phenotypic switching assays were performed as described [3] for a total of 15 passages for strains YJO16 (black bars), YJ-01 (white bars), and YJ-01(pVV60) (gray bars) in LB2 broth supplemented with 1 mM $CaCl_2{\cdot}2H_2O$. Dilutions and platings of the initial overnight culture and following 5, 10, and 15 passages were conducted in order to count and score colony phenotypes. Phenotypes were scored as opaque, translucent, rugose, sectored, which were two phenotypes in one colony, and indeterminant, which included any other uncharacterized phenotype. At each sampling time, the proportions of opaque (O), translucent (T), and rugose (R) colonies to the total number of colonies were calculated and multiplied by 100 for three independent experiments and the means ± SD are presented.

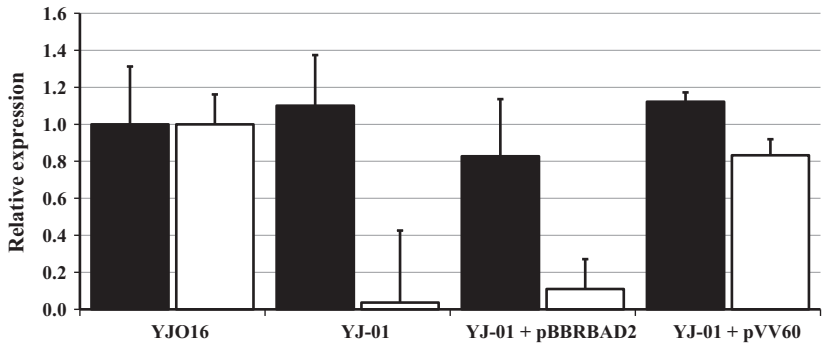

Fig. 3. Reduced distal gene transcription within the Group I CPS operon in the *rfaH* mutant. Total RNA was extracted from mid-exponential cultures of the indicated strains and subjected to RT-qPCR analysis. The graph depicts the expression differences of the *wza* (black bars) and *wbfY* (white bars) genes in YJ-01, YJ-01(pBBRBAD2), and YJ-01(pVV60) relative to their expression in YJ016. All data were normalized with respect to the *tufA* reference gene. Reactions were performed in triplicate and the entire assay was repeated five times with the average relative expressions ± SE being presented here.

plasmid pVV60, but not pBBRBAD2, to YJ-01 restored transcription of *wbfY* to a level that was not significantly different ($P = 0.354$) from that of YJ016 (Fig. 3).

To assess the potential effect on CPS production of reduced distal gene expression of the Group I operon, CPS was extracted from approximately equivalent numbers of cells of these same strains and the amount of galacturonic acid, which has previously been shown to be a component of *V. vulnificus* CPS [21], was quantified by using a colorimetric assay. As shown in Fig. 4A, the quantity of galacturonic acid was negligible for YJ-01 (2.3 ± 2.1 ng·μL^{-1} of extract) relative to YJ016 (66.7 ± 14.4 ng·μL^{-1}) and this difference was considered to be significant ($P < 0.002$). Complementation with pVV60 restored galacturonic acid to

47.5 ± 9.0 ng·μL^{-1}, which was not significantly different from the amount seen for YJ016 ($P = 0.123$). The concentrations of galacturonic acid determined from these assays also correlated with the relative amounts of total CPS present in these same extracts as determined by polyacrylamide gel electrophoresis and subsequent staining with Stains-All (Fig. 4B).

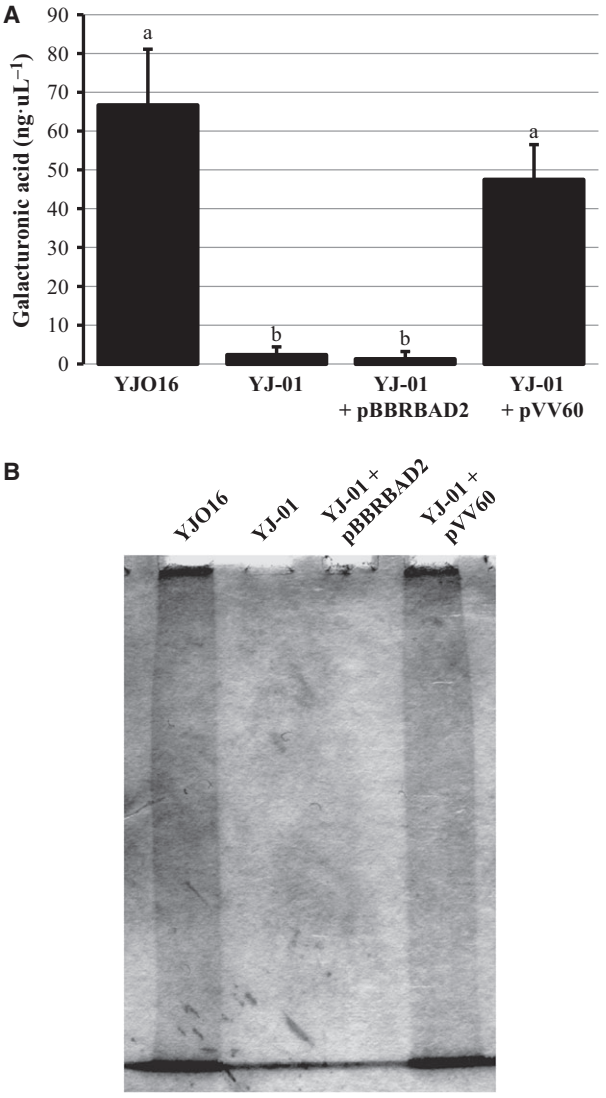

Fig. 4. Decreased CPS production by the *rfaH* mutant. Based on the method described by Lee *et al.* [21], CPS was extracted from approximately equivalent numbers of plate-grown cells of the indicated strains. (A) Galacturonic acid content of CPS extracts. Galacturonic acid content of isolated CPS was determined by using a colorimetric assay [21], and from a total of six assays, the average concentrations of galacturonic acid in ng·μL^{-1} of CPS extract ± SD are presented. (B) Qualitative assessment of CPS concentration in extracts. Equivalent volumes of CPS extracts were electrophoresed on 8% polyacrylamide stacking gels and stained with Stains-All. Two independent isolations of CPS from each strain were performed with approximately the same results in each case.

Loss of RfaH results in extreme serum sensitivity of *V. vulnificus*

Given the role of CPS in serum resistance in *V. vulnificus*, we next subjected these strains to standard serum resistance assays in which approximately 4×10^7 CFU per strain were exposed to NHS for 1 h at 37 °C, and the survivors were then quantified following plating. We found that the *rfaH* mutant YJ-01 was significantly reduced ($P < 0.001$) in serum

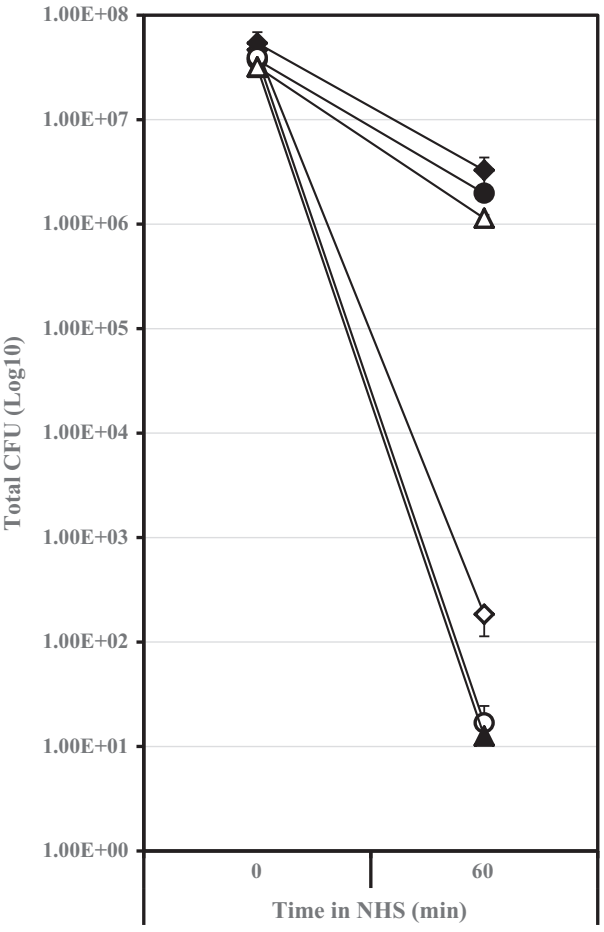

Fig. 5. Sensitivity of the *rfaH* mutant to normal human serum. Equivalent aliquots of each of the indicated strains were either serially diluted and plated to determine CFUs at time zero or they were mixed with normal human serum, and after 60 min of incubation, the reactions were serially diluted, plated, and the surviving CFUs were determined. The assay was repeated at least three times for each strain and the geometric means ± SD are presented. Symbols: (●), YJ016; (○), YJ-01; (▲), YJ-01 (pBBRBAD2); (△), YJ-01(pVV60); (♦), 1003(O); (◇), ABZ1(T).

resistance by approximately 5 orders of magnitude relative to the parent YJ016 (Fig. 5). As expected, addition of pVV60, but not pBBRBAD2, restored high-level resistance of YJ-01. The results here contrasted sharply with those obtained in control assays in which NHS was replaced by heat-inactivated human serum or PBS; in those cases, the mutant YJ-01 survived as well as YJ016 (data not shown), which is consistent with the view that YJ-01 was sensitive to the active complement system present in NHS.

To provide further context regarding the sensitivity of strain YJ-01 to NHS, we compared its results to another translucent strain ABZ1(T), which is a derivative of opaque clinical isolate 1003(O). Strain ABZ1(T) contains a previously described transposon insertion in an epimerase gene that was shown to be essential for CPS production [24]; moreover, ABZ1(T) was found to be greatly attenuated, such that in multiple studies, it did not kill any iron-loaded mice at doses as high as 4.9×10^7 CFU [6,24]. Here we found that while the parental strain 1003(O) showed resistance to NHS at a level similar to YJ016, ABZ1(T) was reduced significantly in resistance ($P < 0.001$) by approximately 4 orders of magnitude (Fig. 5). Although ABZ1(T) typically yielded somewhat more survivors than YJ-01 following exposure to NHS, we found no significant difference overall between them in these assays ($P = 1.000$); thus, we conclude that the *rfaH* mutant YJ-01 of *V. vulnificus* is as sensitive to serum killing as the potentially avirulent and acapsular translucent strain ABZ1(T).

Discussion

Although RfaH antitermination appears to be a well-conserved mechanism among Gram-negatives, the specific secreted factors and surface molecules controlled by RfaH vary from species to species. Here, we assessed the effects of *rfaH* gene inactivation in *V. vulnificus* on expression of the Group I CPS operon and found conclusive evidence that RfaH plays a significant role in CPS production and serum resistance in this bacterium. A considerable number of virulence factors have been identified in *V. vulnificus*, including siderophores, LPS, CPS, pili, flagella, certain outer membrane proteins and several toxins; however, due to its central role in protecting the bacterium from the host immune response, CPS is one of the few factors recognized as being absolutely essential for pathogenicity [2,25,26]. Our results here suggest that the RfaH protein itself is also essential for the ability of *V. vulnificus* to cause disease because of its critical role in transcription of CPS genes.

There is at least one additional target of RfaH in *V. vulnificus* as an *ops* element was also identified within the *brp* operon [6]. This set of genes encodes functions required for rugosity and production of the exopolysaccharide associated with rugose cells, and transcription of the *brp* genes was found to be highly up-regulated in rugose compared to opaque and translucent variants [6,27]. Eventual characterization of RfaH control of rugosity in *V. vulnificus* would require construction of an *rfaH* mutant beginning with a rugose parent rather than the opaque one used here and assessment of the effects of this defect on *brp* operon transcription and rugose exopolysaccharide production. The potential for reduced distal gene transcription within both the Group I CPS and *brp* operons in *rfaH* mutant cells may explain why we did not observe switching here of strain YJ-01 to either opaque or rugose.

An additional polysaccharide controlled by RfaH in some bacteria is the O-antigen of LPS, and, like CPS, O-antigen is considered to be a major contributor to serum resistance [28]. While a role for O-antigen in serum survival of *V. vulnificus* does seem likely [29], the potential for *rfaH* control of O-antigen production in *V. vulnificus* is at present unclear as little is known about the genetic determinants of LPS synthesis in this organism [29–31].

There have been a number of efforts over the years aimed at developing effective vaccines for *V. vulnificus*, including recent attempts involving either subunit or whole-cell candidates [32–35]. A recently described attenuated mutant defective for production of three different cytotoxins resulted in an induced antibody response to *V. vulnificus* and provided protection in mice against challenge with a virulent strain [32]. In the last decade, the use of *rfaH* mutants as a basis for live attenuated *Salmonella enterica* vaccine strains has shown promise [36–40]. Our results here demonstrating that strain YJ-01 was defective for CPS production raise the possibility that a *V. vulnificus rfaH* mutant may also prove to be an effective attenuated vaccine candidate. This assertion is bolstered by evidence that loss of CPS by *V. vulnificus* not only results in attenuation of virulence but also likely unmasks other immunogenic surface components of this pathogen [1,2].

Acknowledgements

We thank Jennifer Carstens for construction of plasmids pVV37 and pVV38 and Zelam Kaluskar for help with southern blotting. We are also grateful to Bliss Lambert for assistance with preparation of certain

figures. This work was supported in part by the Louisiana Sea Grant College Program with funds from the National Oceanic and Atmospheric Administration Office of Sea Grant, Department of Commerce, under grant no. NA06OAR4170022, project no. R/PMO-21 and Louisiana State University. Statements, findings, conclusions, and recommendations are those of the authors and do not necessarily reflect the views of either the Louisiana Sea Grant College Program or the U.S. Department of Commerce.

Author contribution

KLGS and GSP conceived the study; SBG, KLGS, and GSP designed the experiments; SBG and KLGS performed the experiments; SBG, JTC, and GSP analyzed the data; SBG and GSP wrote the manuscript with input from JTC.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Growth curves of strains YJO16, YJ-01, YJ-01(pBBRBAD2), and YJ-01(pVV60).