Role of GacA in virulence of *Vibrio vulnificus*

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The GacS/GacA two-component signal transduction system regulates virulence, biofilm formation and symbiosis in *Vibrio* species. The present study investigated this regulatory pathway in *V. vulnificus*, a human pathogen that causes life-threatening disease associated with the consumption of raw oysters and wound infections. Small non-coding RNAs (csrB1, csrB2, csrB3 and csrC) commonly regulated by the GacS/GacA pathway were decreased (P<0.0003) in a *V. vulnificus* CMCP6 ΔgacA::aph mutant compared with the wild-type parent, and expression was restored by complementation of the gacA deletion mutation in trans. Of the 20 genes examined by RT-PCR, significant reductions in the transcript levels of the mutant in comparison with the wild-type strain were observed only for genes related to motility (flaA), stationary phase (rpoS) and protease (vvpE) (P=0.04, 0.01 and 0.002, respectively). Swimming motility, flagellation and opaque colony morphology indicative of capsular polysaccharide (CPS) were unchanged in the mutant, while cytotoxicity, protease activity, CPS phase variation and the ability to acquire iron were decreased compared with the wild-type (P<0.01). The role of gacA in virulence of *V. vulnificus* was also demonstrated by significant impairment in the ability of the mutant strain to cause either skin (P<0.0005) or systemic infections (P<0.02) in subcutaneously inoculated, non-iron-treated mice. However, the virulence of the mutant was equivalent to that of the wild-type in iron-treated mice, demonstrating that the GacA pathway in *V. vulnificus* regulates the virulence of this organism in an iron-dependent manner.

**INTRODUCTION**

*Vibrio vulnificus* is a moderately halophilic, Gram-negative bacterium that inhabits coastal waters and colonizes fish and filter-feeding shellfish (DePaola et al., 1994; Motes et al., 1998; Tamplin & Capers, 1992; Wright et al., 1996). Individuals with underlying conditions such as liver disease, diabetes mellitus, cancer, haemochromatosis (iron-overload) or immune system dysfunction can incur life-threatening systemic disease from consumption of raw oysters or from wound infections (Blake et al., 1979; Jones & Oliver, 2009). The virulence of this bacterium in animal models has been attributed to multiple factors (Gulig et al., 2005; Jones & Oliver, 2009), including capsular polysaccharide (CPS) expression (Amako et al., 1984; Kreger et al., 1984; Simpson et al., 1987; Yoshida et al., 1985), iron acquisition (Litwin et al., 1996; Simpson & Oliver, 1983; Wright et al., 1986), pili (Gander & LaRocco, 1989; Paranjpye & Strom, 2005; Paranjpye et al., 2007), flagella (Kim & Rhee, 2003; Lee et al., 2004), quorum sensing (McDougald et al., 2001) and cytolytic haemolysin encoded by the rtxA1 gene (Lee et al., 2007; Kim et al., 2008; Liu et al., 2007). Other cytotoxin/haemolysin genes, including rtxA2 and rtxA3 (J. L. Joseph and P. A. Gulig, unpublished results) and vvhA (Wright & Morris, 1991), as well as a metalloprotease encoded by vvpE (Jeong et al., 2000; Shao & Hor, 2000), show no apparent role in the virulence of this bacterium in the mammalian system. CPS is perhaps the most definitive virulence factor, and phase variation of CPS is correlated with changes in colony morphology, whereby opaque (Op) strains are encapsulated and virulent, while translucent (Tr) variants show reduced encapsulation and virulence. Both colony types undergo reversible phase variation to the alternate phenotype.
We hypothesized that phenotypic traits that contribute to the virulence and survival of *V. vulnificus* are controlled by the GacS/GacA system. Therefore, a gacA deletion mutation was constructed in a clinical strain of *V. vulnificus* and examined for altered phenotypes and differential gene expression relative to the wild-type.

**METHODS**

**Bacterial strains and growth assays.** Bacteria were stored at −80 °C in 50 % (v/v) glycerol in Luria–Bertani broth with NaCl (LBN) prepared with 1 % tryptone, 0.5 % yeast extract and 1 % NaCl at pH 7. Isolated colonies were recovered on LBN agar (LBNA), prepared with 1.5 % agar and with or without antibiotics, as described below. Media were supplemented with 1 µg tetracycline ml⁻¹ to maintain the plasmids for all in vitro phenotypic assays. Electrocompetent *E. coli* EC100D TransformMax cells (Epicentra Biotechnologies) and *E. coli* SI7-1 zipr (Simon et al., 1983) were used for cloning and complementation. Unless otherwise stated, media components were purchased from Difco, and chemicals were purchased from Sigma.

**Construction and complementation of a *V. vulnificus gacA* deletion/insertion mutant.** Genomic DNA was isolated from overnight cultures of *V. vulnificus* CMCP6 using the UltraClean Microbial DNA Isolation kit (Mo Bio Laboratories). Plasmids were purified using the QiAprep Spin Miniprep kit (Qiagen). Standard molecular biology procedures were used for PCR verification of constructs, plasmids and genomic regions. USER (uracil-specific excision reagent) Friendly Cloning (New England Biolabs) was used to construct a deletion mutation by directionally cloning 500 bp amplicons of flanking DNA from the *V. vulnificus* gacA locus (Gulig et al., 2009). An Smal site was included in the gacA upstream and downstream primers for blunt-end insertion of a kanamycin-resistance marker between the joined flanking regions. The deletion construct was created in pGTR1129, a broad-host-range plasmid modified with USER ends (Gulig et al., 2009). The suicide vector was linearized by mechanical shearing and transformed into *V. vulnificus* via chlorin induction (Gulig et al., 2009; Meibom et al., 2005). V. vulnificus AgaA::aph mutants were selected on LBNA with 100 µg kanamycin ml⁻¹. Homologous recombination into the *V. vulnificus* genome at gacA flanking regions was confirmed by sequencing [Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida] PCR products of both the mutant and wild-type using primers (GenoMechanix) to genomic regions outside the cloned flanking regions.

**RNA transcript analysis.** Isolated colonies were inoculated into 5 ml LBN and incubated overnight at room temperature without shaking. Starter cultures were diluted to OD₆₅₀ 0.01 and incubated with aeration at 37 °C for 18–20 h (OD₆₅₀ ~ 1.5). Quadruplicate technical replicates of each biological sample (*n* = 3) were incubated for 5 min in a 2 × volume of RNAprotect Bacteria Reagent (Qiagen), centrifuged at 5000 × g for 10 min, and stored at −70 °C for subsequent isolation of total RNA using RNeasy Mini (Qiagen) and Turbo RNA-free kits (Ambion). Total RNA concentration and quality were determined by *A₂₆₀/A₂₈₀* values on a NanoPhotometer (Implen) and were verified by agarose gel electrophoresis. RNA (2 µg) was converted to cDNA by random priming using a SuperScript VILO cDNA Synthesis kit (Invitrogen), and cDNA was diluted 1:20 for real-time PCR. Controls without reverse transcriptase were used to determine the level of genomic DNA contamination. Specific primers were synthesized by GenoMechanix, using previously described sequences for csrB1, csrB2, csrB3 and csrC (Kulkarni et al., 2006). Primer 3 (Rozen & Skaletsky, 2000) was used to design primers for putative virulence and survival genes and for the internal control gene *tufA*. Reactions were conducted on a SmartCycler II thermocycler (Cepheid) in a 20 µl volume with 0.4 µl of primers (10 mM) and 2 µl template using the EXPRESS SYBR GreenER qPCR SuperMix Universal system (Invitrogen). Internal control and target gene primers were confirmed to be between 90 and 110 % efficient for fold-difference determination. Mel peaks based on the first derivative were used to confirm the sequence identity of amplicons.

**Reporter construction and bioassay for csrB*-luxABCDE*.** The predicted promoter region for *V. vulnificus* CMCP6 csrB1 was
amplified by PCR using forward and reverse primers (5'-TTC-AAGCCACCGCTCAGGTCTTGA-3' and 5'-GGTTGCTGTGTTGGTGGTGGG-3'), respectively. The resulting ~450 bp product was cloned into the pCR-BluntII-TOPO vector (Invitrogen), and subsequently excised with EcoRI and subcloned into EcoRI-digested and calf intestinal alkaline phosphatase-treated pSB401 (Winson et al., 1998) to construct the pMT39 promoter-reporter with P_{arB1}-luxCDABE. The orientation of the insert in pMT39 was confirmed by PCR. The pMT42 (P_{arB2}-luxCDABE) and pMT40 (P_{uvrY}-luxCDABE) plasmids were constructed similarly using forward and reverse primers (5'-TCTTGGTGGTGGTGGTGGG-3' and 5'-GGTTGCTGTGTTGGTGGTGGG-3') and EcoRI-digested pSB401 and pSB402 digested with NcoI and XhoI, respectively. The resulting ~450 bp product was cloned into the pCR-BluntII-TOPO vector (Invitrogen), and confirmed by sequencing. pMT39, pMT40, and pMT42 were transformed into E. coli MG1655 and its isogenic uvrY (gacA orthologue) negative derivative, RG133 (Goodier & Ahmer, 2001). Luminescence of the reporters was measured with a Victor3 multimode microtitre plate reader (PerkinElmer) in black clear-bottomed plates, as described previously (Teplitzki et al., 2006).

The V. vulnificus CMP6 gacA gene with its own ribosome-binding site was amplified by PCR catalysed by Taq (New England Biolabs) using primers CJK57 (5'-AAGCCACCGCTCAGGTCTTGA-3') and CJK58 (5'-GGTTGCTGTGTTGGTGGTGGG-3') and engineered HindIII sites (underlined). The resulting 758 bp PCR product was cloned into pCR2.1-TOPO (Invitrogen) and confirmed by sequencing (ICBR, University of Florida) with standard primers M13R and M13F. The product was subcloned into the HindIII site of pBAD18-Ap (Guzman et al., 1995), which offers tight regulation and high-level expression via the araBAD promoter, yielding pCJ5K, which was then transformed into chemically competent E. coli DH5α. Transformants were confirmed to carry the plasmid by antibiotic selection and by PCR with MT13 (5'-ACCTTGCTATGCCATATGATAGCTTACGACGG-3') and CJK58 primers. Plasmid pCJ5K was then electroporated using a Bio-Rad MicroPulser (Bio-Rad Laboratories) into electroporoent competent cells of either wild-type E. coli MG1655 or its isogenic uvrY33::kan derivative E. coli RG133, containing pMT39, pMT40 or pMT42, as described above. The pBAD18-Ap vector was also transformed into MG1655 or RG133 containing the promoter constructs.

**Colony morphology and phase variation.** Op versus Tr colony morphology was determined on LBNA after 24 h incubation at 37 °C, as previously described (Chatzidaki-Livanis et al., 2006a). To examine CPS phase variation, isolated colonies were inoculated into 5 ml LB broth and shaken overnight at 37 °C. Replicate aliquots of cultures (n=3) were washed three times (10,000 g 30 s) in PBS and diluted to 10^{-6} c.f.u. ml^{-1}. Inocula (1 ml) were diluted to 5 ml 1% Protease Peptone No. 3 (PP3) in PBS (pH 7.0) in a 250 ml flask. Cultures were incubated statically at 37 °C, streaked to duplicate LBNA plates after 7 days and incubated overnight at 37 °C, and phase variation was determined from the percentage of Tr colonies. The results are the composite of two independent experiments.

**Motility assay.** Isolated colonies were grown statically overnight at room temperature in LB, diluted 1:10 with fresh LB, and shaken at 37 °C for approximately 2 h to achieve a cell density of 10^{8} c.f.u. ml^{-1} (OD_{600} 0.30) to generate exponential phase cells. Stationary phase cells were taken directly from overnight cultures grown at 37 °C. For each strain, six replicates were examined on LBNA or LBNA without yeast extract (LBNA/-Y) and on tryptic soy agar (TSA) plates that were prepared with 0.3, 0.5 or 0.7% (w/v) agar. Plates were spotted with 3 μl of either exponential or stationary phase culture and incubated at 30 or 37 °C. The diameter of the cell spreading was measured as an indicator of motility after 13, 21 and 24 h. Flagellar morphology was examined using NanoOrange stain (Invitrogen) on bacteria recovered from 3% TSA plates (Grossart et al., 2000), as modified by Gulgil et al. (2009).

**Protease assay.** Strains taken from frozen stock were inoculated into fresh LBN (with 1 μg tetracycline ml^{-1} where appropriate) and grown to OD_{600} 1–2. Cultures were then centrifuged at 4 °C at 6000 g for 30 min. The supernatant was filter-sterilized using a 0.2 μm pore-size filter and used as a crude enzyme extract for measurement of protease activity, as described elsewhere (Miyoshi et al., 1987). Azocasein solution (200 μl at 5 mg ml^{-1} in 0.02 M Tris/ HCl buffer, pH 7.5) was mixed with 400 μl crude enzyme preparation and incubated at 37 °C for 1 h. To stop the reaction, 1.4 ml 5% TCA was added, and the reaction was centrifuged at 1000 g for 5 min. Supernatant (1 ml) was mixed with an equal volume of NaOH (0.5 M), and A_{440} was measured.

**Cytotoxicity to INT-407 cell monolayers.** RtxA1 activity was assessed by detachment of INT-407 cell monolayers, as previously described (Jeong et al., 2000). Briefly, INT-407 cells were seeded in a 24-well tissue culture plate for 2 days until monolayers reached 80–90% confluency. Exponential phase V. vulnificus cultures were diluted in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), and approximately 10^{6} c.f.u. of V. vulnificus was added to each well for an m.o.i. of 10. Triplicate infections were used for each strain. Uninfected cells and wells with media alone were used as negative controls for lysis and background staining of wells, respectively. Gentamicin (100 μg ml^{-1}) was added 1 h after infection, and the unwashed cells were incubated overnight. After overnight incubation, the wells were washed twice with PBS, and the cells remaining attached to the wells were stained with 0.05% (w/v) crystal violet for 10 min at room temperature, washed four times with PBS, and the crystal violet was extracted with 95% ethanol. The crystal violet/ethanol solution (150 μl) was transferred to a 96-well plate, and the A_{490} was measured using an ELx808UV microplate reader (Bio-Tek Instruments). The percentage monolayer detachment was calculated by subtracting the A_{490} of the medium-only control from the A_{490} for each infected and uninfected well. The ratio of attached infected cells to attached uninfected cells was calculated from the adjusted A_{490} for each infected well divided by the adjusted A_{490} of uninfected cells (mean of all three wells), and was converted to percentage detachment/destruction by the formula: 100−(ratio × 100).

**Iron-limited growth.** Bacterial inocula were prepared from overnight cultures grown at 37 °C and were diluted to OD_{600} 0.3 in medium. Tetracycline (1 μg ml^{-1}) was added to maintain plasmids where appropriate. Cells were washed twice in LBN and suspended in 1 ml LBN. For iron-limited growth, inocula were diluted 1:50 in LBN (35 ml) with 150 μM dipiridyl and incubated at 37 °C. The percentage growth yield was determined by OD_{600} and by plate counts on LBNA, and the percentage growth yield was calculated as growth in LBN with dipiridyl divided by growth in LBN (ratio × 100).

**Mouse virulence.** The virulence of V. vulnificus in terms of systemic and localized infection was determined using a previously described mouse model (DePaola et al., 2003). Seven- to 10-week-old female ICR mice (Harlan Sprague–Dawley) (n=5) were used for all experiments. Iron-treated mice were injected intraperitoneally with 250 μg iron dextran per gram body weight, while non-iron-treated mice did not receive exogenous iron. Bacteria in 0.1 ml PBS were obtained from exponential phase cultures and were injected subcutaneously (s.c.), using approximately 10^{7} c.f.u. for injections in iron-treated mice and 10^{6} c.f.u. for injections in mice that did not receive iron. Mice were euthanized by CO2 asphyxiation when rectal temperatures dropped below 35 °C or at 20 h post-infection. Bacterial infection of skin tissue (local infection) and liver tissues (systemic infection) was quantified by homogenization of tissues, dilution and plating on LBNA. Skin lesion c.f.u. and liver c.f.u. values were log_{10}-transformed for statistical analysis.
Statistics. Significant differences in the phenotypes of the wild-type versus the mutant were determined by Student's $t$ test (two-tailed, type 2). Transcript fold differences between mutant and wild-type strains were determined using the $2^{ΔΔCt}$ method (Livak & Schmittgen, 2001) and were based on the mean of three biological samples (individual RNA extracts). Significant differences between wild-type and mutant strains were calculated by Student's $t$ test, comparing $ΔC_{t}$ values (target gene $C_{t}$—reference $C_{t}$) of the wild-type versus the mutant, and fold difference was based on $ΔΔC_{t}$ (mutant mean $ΔC_{t}$—wild-type mean $ΔC_{t}$) and calculated as $2^{ΔΔC_{t}}$.

RESULTS

The GacS/GacA pathway of V. vulnificus

In order to understand the role of the GacA protein in gene expression in V. vulnificus, a deletion of gacA marked with a kanamycin-resistance gene cassette ($ΔgacA::aph$) was constructed in CMCP6, as described in Methods. Strains and plasmid constructs are described in Table 1. The function of the GacA pathway was compared in the mutant and wild-type strains by quantitative RT-PCR of the sRNAs that are predicted to bind to the CsrA protein in V. vulnificus, namely csrB1, csrB2, csrB3 and csrC (Kulkarni et al., 2006). In the stationary phase of growth (Table 2), the $ΔgacA::aph$ mutant showed significantly reduced expression for sRNAs csrB1 and csrC (>1100-fold) and to a lesser extent for csrB2 (235-fold) and csrB3 (18-fold) compared with the wild-type ($P=2 \times 10^{-6}$, $2 \times 10^{-6}$, $8 \times 10^{-5}$ and $2 \times 10^{-4}$, respectively). Defects in sRNA synthesis in the $ΔgacA::aph$ mutant were complemented in trans by introduction of the gacA gene on a plasmid, but the vector control did not restore expression. The fold differences in gene expression for csrB2 (1.71) and csrB3 (0.86) were not significantly different between the complemented and wild-type strains. Although the fold difference in gene expression between these strains remained significant ($P≤0.003$) for both csrB1 (8.92) and csrC (10.51), fold differences were also significantly different ($P<0.0003$) for the complemented mutant compared with the plasmid control (8.65 and 71.18, respectively). It should be noted that the fold differences observed between the complemented and wild-type strains were greatly reduced compared with the >1100-fold difference observed between the mutant and wild-type strains. No significant differences in gene expression were observed between mutant and wild-type strains when RNA was harvested during exponential phase growth (not shown).

V. vulnificus PcsrB :: luxCDABE promoter fusions were also used in an S. enterica background to examine the effects of GacA on gene expression (not shown). Expression of V. vulnificus PcsrB :: luxCDABE was minimal in a Salmonella strain without a functional orthologous gacA gene (sirA3 :: cat); however, in the wild-type S. enterica, expression was similar to that of an E. coli PcsrB :: luxCDABE in the same strain. GacA regulation of V. vulnificus csrB1, csrB2 and csrB3 promoters was also confirmed by the introduction of the V. vulnificus gacA gene under the control of an arabinose-inducible promoter into a gacA-negative background in E. coli RG133. Introduction of the V. vulnificus GacA increased expression of the lux reporter gene fusions for V. vulnificus csrB1 and csrB2 promoters by about five- and 1000-fold, respectively (Fig. 1). However, no increase was observed for csrB3. As expected, regulation of sRNAs remained at the basal level when grown under

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>V. vulnificus strains</strong></td>
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<td>CMCP6</td>
<td>Wild-type clinical isolate, virulent, encapsulated Op colony, biotype 1</td>
<td>Kim et al. (2003)</td>
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<td>CMCP6 $ΔgacA::aph$</td>
<td>gacA deletion mutant with kanamycin-resistance gene inserted in the deletion site</td>
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<td><strong>E. coli strains</strong></td>
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<td><strong>Plasmids</strong></td>
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Table 2. Comparison of transcript levels in V. vulnificus CMCP6 and in the ΔgacA::aph mutant

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<th>Function</th>
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<th>Reverse primer sequence (5′–3′)</th>
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<td>ttttacgcctcactcctcct</td>
<td>1.16 (NS)</td>
</tr>
<tr>
<td>smcR</td>
<td>LuxR homologue</td>
<td>ttgcggtgctgatgtgcagtctc</td>
<td>ttttacgcctcactcctcct</td>
<td>0.52 (NS)</td>
</tr>
<tr>
<td>tufA</td>
<td>Elongation factor</td>
<td>ttgcaggttgagcagcagataaat</td>
<td>cgggattgagaggtgagagaa</td>
<td>N/A‡</td>
</tr>
<tr>
<td>vvhA</td>
<td>Haemolysin</td>
<td>ttgcaggttgagcagcagataaat</td>
<td>cgggattgagaggtgagagaa</td>
<td>0.22 (NS)</td>
</tr>
<tr>
<td>vvpE</td>
<td>Protease</td>
<td>cagacgctactcctctctctcctct</td>
<td>ttttacgcctcactcctcct</td>
<td>8.5 (0.001)</td>
</tr>
<tr>
<td>wta</td>
<td>CPS transport</td>
<td>agacgctactcctctctctctcctct</td>
<td>ttttacgcctcactcctcct</td>
<td>1.03 (NS)</td>
</tr>
</tbody>
</table>

*The fold decrease in transcript levels for the ΔgacA::aph mutant compared with the wild-type is shown during stationary phase as determined by quantitative RT-PCR. Significant differences (P<0.05) in ΔCt are underlined, and were determined by the ΔΔCt method and are described in Methods.

†NS, No significant differences in ΔCt,
§ND, Not done.
‡NA, Not applicable, as tufA was used for reference gene expression.

In vitro characterization of the gacA mutant

Unlike V. fischeri, for which growth yield is reduced in the gacA mutant (Whistler & Ruby, 2003; Whistler et al., 2007), growth rates and yields of V. vulnificus ΔgacA::aph were virtually identical to those of the wild-type (data not shown). However, the colony morphology of V. vulnificus was similar to that of V. fischeri in that gacA deletion mutants had colonies that were slightly smaller with less yellow pigment than the wild-type strains, and wild-type morphology was restored by complementation (data not shown).

Colony morphology is also indicative of CPS expression in V. vulnificus, and Tr colonies are correlated with decreased virulence (Wright et al., 1990). Upon initial isolation, some ΔgacA::aph isolates appeared to have mixed Op and Tr colonies, and the presence of the gacA deletion mutation was confirmed by DNA sequencing in both Op and Tr colony types. Therefore, Tr morphology was assumed to be the consequence of spontaneous phase variation that occurred during the process of constructing the deletion
mutations. Although the morphology of Op ΔgacA::aph isolates proved to be stable upon further isolation during standard subculture, the frequency of phase variation in the mutant compared with the wild-type strain was further investigated, using previously described conditions that enhance the rate of the Op to Tr phase shift (Chatzidaki-Livanis et al., 2006b). After 7 days in PP3 medium, the mutant was significantly (P<0.001) reduced for phase variation compared with the wild-type strain, as evidenced by the recovery of only 0.4 ± 0.5 % Tr colonies from the mutant and 29.6 ± 2.9 % Tr colonies from the parent strain (Table 3). Complementation of the ΔgacA::aph mutation in trans restored the frequency of Tr colonies to wild-type levels, while the vector control did not.

RtxA1 cytotoxicity contributes to the virulence of V. vulnificus in mice, and RtxA1 activity is indicated by destruction of V. vulnificus INT-407 monolayers (Lee et al., 2003). The deletion of luxCDABE from V. vulnificus expressed from an arabinose-inducible promoter on pCJK57 (see Table 1). Strains contained promoter reporters either with pCJK57 in the presence of arabinose (○) or without pBAD18 vector control in the presence of arabinose (●), or without pCJK57 in the presence of arabinose (▲). The substitution of glucose for arabinose, as expected, eliminated complementation by gacA borne on pCJK57 (data not shown).

**Mouse virulence**

Subcutaneous inoculation was used in a mouse model to examine localized skin infection, as measured by c.f.u. per gram of skin tissue following infection, and systemic disease, as determined by c.f.u. per gram of liver. Decreased temperature indicates systemic infection, and a rectal temperature below 33 °C is a surrogate for death (DePaola et al., 2003). The deletion of gacA impaired the capacity of V. vulnificus to cause both localized and systemic infections in mice, and this defect was related to iron

![Expression of PcsrB1v-luxCDABE](image_url)

**Expression of V. vulnificus PcsrB-luxCDABE promoters in a heterologous host.** To reconstruct the GacA–csrB pathway of V. vulnificus, promoters for V. vulnificus CMCP6 (a) csrB1, (b) csrB2 and (c) csrB3 sRNAs were cloned upstream of a promoterless luxCDABE cassette. Regulation of the reporters was tested in an E. coli MG1655 uvrY33::Tn5 mutant (gacA orthologous mutant) in the presence of gacA from V. vulnificus expressed from an arabinose-inducible promoter on pCJK57 (see Table 1). Strains contained promoter reporters either with pCJK57 in the presence of arabinose (●) or with pBAD18 vector control in the presence of arabinose (○), or without pCJK57 in the presence of arabinose (▲). The substitution of glucose for arabinose, as expected, eliminated complementation by gacA borne on pCJK57 (data not shown).
pretreatment of the host. Lesions that were apparent following wild-type infections were not evident or were greatly reduced in non-iron-treated mice that were infected with the *V. vulnificus gacA* mutant (Fig. 3). The c.f.u. g$^{-1}$ recovered from both skin ($P=0.0003$) and liver ($P=0.017$) tissues was also significantly lower in the mutant compared with the wild-type (Table 4). Higher body temperatures were observed with the mutant compared with wild-type infections, but differences were not significant. Conversely, the mutant was not affected for any measure of virulence in

**Table 3. Phenotypic characterization of the gacA mutant**

<table>
<thead>
<tr>
<th>V. vulnificus strain</th>
<th>Phase variation$^*$ (percentage Tr)</th>
<th>Motility zone diameter$^+$ (mm)</th>
<th>Cytotoxicity$^\ddagger$ (percentage destruction of monolayer)</th>
<th>Protease activity$^\S$ ($A_{440}$)</th>
<th>Iron response$^|$ (percentage growth yield)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMCP6</td>
<td>29.6 ± 2.9</td>
<td>18.8 ± 0.2</td>
<td>80 ± 8.9</td>
<td>0.31 ± 0.054</td>
<td>84.1 ± 0.14</td>
</tr>
<tr>
<td>CMCP6 ΔgacA</td>
<td>0.4 ± 2.9</td>
<td>22.7 ± 0.7</td>
<td>ND$^\ddagger$</td>
<td>0.05 ± 0.005</td>
<td>8.7 ± 0.11</td>
</tr>
<tr>
<td>CMCP6 ΔgacA (pGacA)</td>
<td>27.0 ± 1.8</td>
<td>17.7 ± 0.6</td>
<td>77.2 ± 10.36</td>
<td>0.07 ± 0.035</td>
<td>70.7 ± 0.31</td>
</tr>
<tr>
<td>CMCP6 ΔgacA (pGTR116)</td>
<td>0.8 ± 0.7</td>
<td>ND$^\ddagger$</td>
<td>44.6 ± 3.97</td>
<td>0.01 ± 0.003</td>
<td>2.9 ± 0.02</td>
</tr>
</tbody>
</table>

$^*$Phase variation was measured as the percentage of Tr colonies recovered on LBNA after 7 days incubation in PP3 medium from triplicate samples. Significant ($P<0.0001$) differences noted for the mutant and vector control compared with the wild-type are underlined. Results represent two experiments ($n=4$).

$^+$Motility was measured on LBNA incubated at 30 °C for 13 h, and significant differences ($P<0.01$) compared with the wild-type are underlined.

$^\ddagger$Rtx toxicity was examined by destruction of INT-407 intestinal epithelial cell line monolayers using the ratio of attached infected cells to attached uninfected cells, as calculated from the adjusted $A_{490}$ for each infected well divided by the adjusted $A_{490}$ of uninfected cells (mean of all three wells) and converted to percentage detachment/destruction of attached cells from the monolayer by the formula: ratio $\times$ 100. Significant differences are underlined for $P<0.003$ among triplicate samples.

$^\S$Protease activity was examined by degradation of azocasein measured at $A_{440}$, and significant differences ($P<0.01$) are underlined.

$^\|$Iron response was determined by percentage growth yield as measured by plate counts on LBNA from cultures grown in LB with or without dipyridyl (150 μM), and was calculated as LBN + dipyridyl growth/LBN growth (ratio $\times$ 100) from the mean of triplicate samples. Significant differences ($P<0.01$) are underlined.

$^\ddagger$ND, Not done.

![Fig. 2. Morphology of V. vulnificus ΔgacA::aph versus that of the wild-type on motility agar. Representative growth of V. vulnificus CMCP6 on TSA motility agar (0.3 %) after incubation at 30 °C for 24 h. Plates are shown for (a) wild-type, (b) ΔgacA::aph (ΔgacA), (c) the complemented mutant [ΔgacA (pGacA)] and the vector control [ΔgacA (vector)].](image-url)
mice that were pre-treated with iron dextran. Complementation of the ΔgacA::aph mutation with pGacA mostly restored the level of skin infection, but did not significantly change the levels of liver infection or temperature caused by the mutant strain.

**DISCUSSION**

This study is believed to be the first report of GacA function in *V. vulnificus*. GacA transduces signals from GacS in γ-proteobacteria to directly increase expression of sRNAs that in turn sequester the global regulatory protein CsrA (Baker et al., 2002; Wei et al., 2001). Our results are consistent with prior descriptions of GacS/GacA regulation in other bacteria, and the regulatory pathway in *V. vulnificus* CMCP6 was confirmed by observed decreases in relevant sRNA transcripts in a ΔgacA::aph mutant compared with the parental wild-type strains and by complementation of the mutant with the gacA gene presented in trans. Loss of gacA in the mutant made csrB1 and csrC transcripts essentially undetectable, but had

**Table 4. Role of GacA in virulence of *V. vulnificus***

<table>
<thead>
<tr>
<th>Strain</th>
<th>Non-iron-treated</th>
<th>Mouse virulence*</th>
<th>Iron-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Skin infection</td>
<td>Liver infection</td>
<td>Temperature</td>
</tr>
<tr>
<td></td>
<td>[log(c.f.u. g⁻¹)]</td>
<td>[log(c.f.u. g⁻¹)]</td>
<td>(°C)</td>
</tr>
<tr>
<td>CMCP6</td>
<td>7.4±0.3</td>
<td>4.7±1.1</td>
<td>35.0±3.1</td>
</tr>
<tr>
<td>CMCP6 ΔgacA</td>
<td>3.9±1.3</td>
<td>2.8±0.9</td>
<td>38.1±0.7</td>
</tr>
<tr>
<td>CMCP6 ΔgacA (pGacA)</td>
<td>6.9±0.8</td>
<td>2.9±1.3</td>
<td>38.4±1.5</td>
</tr>
</tbody>
</table>

*Virulence was assessed in s.c. inoculated mice that either did or did not receive iron dextran and an inoculum of 10⁸ or 10⁶ c.f.u., respectively. Significant differences (underlined) from the wild-type were noted in the recovery of bacteria from both skin (*P*<0.0003) and liver (*P*<0.017) infections, which were restored to wild-type levels by complementation for skin but not liver infection.

†ND, Not done.
lacks albuminase, caseinase and VvpE elastase activity contribute to reduced protease activity, as an Also, decreased expression related to stationary phase (Romeo, 1998). Reduced protease activity related to exponential growth, while promoting gene expression may be a factor contributing to the decreased phase variation that was observed in the gacA mutant. Phase variation is dependent upon the strain and growth conditions and is likely to be a response to rapidly changing environmental conditions (Chatzidaki-Livanis et al., 2006b), Tr strains switch to Op morphology during mouse infections (Wright et al., 1990) and oyster colonization (Srivastava et al., 2009), presumably contributing to survival in the host. In Pseudomonas spp., the GacS/GacA system regulates phase variation to a Tr morphology that enhances host colonization (Han et al., 1997; Sánchez-Contreras et al., 2002). Phase variation to the rugose phenotype in V. cholerae enhances biofilm formation and is mediated through HapR (Yildiz et al., 2004), which is regulated by GacA input to the quorum-sensing system (Lenz et al., 2005). Furthermore, GacA-mediated switching in V. fisheri facilitates a symbiotic

Although the V. vulnificus mutant showed decreased expression of flaA, both motility and flagella expression were maintained compared with the parent strain. Motility on 3% agar was enhanced compared with the wild-type strain at 30 °C but not at 37 °C, demonstrating the complexity and variability of the GacA regulon. The effects of this system on motility are potentially made even more complex by the fact that V. vulnificus possesses six flagellin genes. GacA-regulated motility has been described in several species, but mutants can show either decreased (Duffy & Defago, 2000; Goodier & Ahmer, 2001; Hammer et al., 2002; Kinscherf & Willis, 1999; Teplitski et al., 2003; Wei et al., 2001) or increased motility (Whistler & Ruby, 2003). Although this increased motility has been noted in the related species V. fischeri, gacA mutants were hyperflagellated, which was not the case for V. vulnificus. Additionally, V. vulnificus gacA mutants showed reduced growth on the surface of motility agar plates and failed to form concentric rings on this agar after extended incubation. This ring formation has previously been described as ‘swarming’ in V. vulnificus (Kim et al., 2007); however, this behaviour differs greatly from that of Vibrio paraheamolyticus, which swarms on higher-viscosity agar and uses lateral flagella genes that are not present in V. vulnificus for swarming (Stewart & McCarter, 2003). Therefore, we propose that descriptions of ‘swarming’ in V. vulnificus are not in keeping with the strictest use of the term. The concentric rings of V. vulnificus on low-viscosity agar are more likely related to stationary phase responses to nutrient limitation or perhaps to biofilm formation that occurs at the air–liquid interface. V. vulnificus vvpE mutants described elsewhere are also defective in ring formation (Kim et al., 2007), and since gacA mutants show reduced protease activity and vvpE transcript levels, these defects may also contribute to the differential growth on motility agar.

Although GacA did not modulate capsule as indicated by colony morphology, CPS phase variation was reduced in the V. vulnificus ΔgacA::aph mutant compared with the wild-type. A prior study showed that phase variation is RpoS-dependent in V. vulnificus (Hülsmann et al., 2003), and decreased rpoS expression may be a factor contributing to the decreased phase variation that was observed in the gacA mutant. Phase variation is dependent upon the strain and growth conditions and is likely to be a response to rapidly changing environmental conditions (Chatzidaki-Livanis et al., 2006b), Tr strains switch to Op morphology during mouse infections (Wright et al., 1990) and oyster colonization (Srivastava et al., 2009), presumably contributing to survival in the host. In Pseudomonas spp., the GacS/GacA system regulates phase variation to a Tr morphology that enhances host colonization (Han et al., 1997; Sánchez-Contreras et al., 2002). Phase variation to the rugose phenotype in V. cholerae enhances biofilm formation and is mediated through HapR (Yildiz et al., 2004), which is regulated by GacA input to the quorum-sensing system (Lenz et al., 2005). Furthermore, GacA-mediated switching in V. fisheri facilitates a symbiotic

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lifestyle (Whistler et al., 2007). Thus, phase variation appears essential to the life cycles of these bacteria, and these transitions are likely needed for infection/colonization of both mammalian and molluscan hosts by V. vulnificus.

Discrepancies were found in correlating some of the defective phenotypes of the mutant with the corresponding gene expression or with complementation in trans. These anomalies may reflect the complexity of the GacA pathway. Plasmid instability, gene dosage or post-transcriptional modulation may also be contributing factors. A complication of the GacA-mediated response is that CsrA itself somehow activates expression of the sRNAs in the pathway to provide a feedback loop for homeostatic regulation that can be independent of GacA (Suzuki et al., 2002). Furthermore, as shown in the same report, both barA (gacA orthologue) deletion mutation and overexpression of barA result in decreased CsrA-mediated biofilm formation in E. coli. Thus, the autoregulatory aspects of CsrA may complicate the expected response for GacA mutants and complemented mutants. CsrA binding to mRNA can either destabilize (Baker et al., 2002) or stabilize transcripts through mechanisms that are not completely understood (Wei et al., 2001). Certain transcripts that decay rapidly in csrA+ E. coli strains are completely stable in a csrA− background (Liu et al., 1995), while a csrA mutation causes 10-fold increases in other mRNA targets (ycdT and ydeH) of CsrA (Jonas et al., 2008). Investigations of the GacS/GacA pathway are further confounded by the involvement of multiple global regulators (RpoS, HapR and LuxS). Furthermore, preliminary studies have shown some strain differences in the GacA response of V. vulnificus, and these differences are perhaps not unexpected, as plasticity of the GacA regulon is seen for closely related species (Lapouge et al., 2008).

In summary, the gacA mutant differed greatly from the wild-type in sRNA expression and in multiple phenotypes required for virulence of V. vulnificus, demonstrating the important role of GacA in the biology of this species. Complementation of sRNA defects and for most of the altered phenotypes in the mutant confirmed this role. The signals that trigger the GacS/GacA system in E. coli have recently been shown to be the metabolic end-products formate and acetate, although these signals may vary with species, strain and growth conditions (Gonzalez Chavez et al., 2010). Further studies are needed to sort out signals, targets and alternative pathways within different species, and perhaps even different strains of bacteria that are dependent upon GacA for survival.

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