Defects in ex vivo and in vivo growth and sensitivity to osmotic stress of group A Streptococcus caused by interruption of response regulator gene vicR

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The regulator VicR of the two-component regulatory system VicRK is essential in several Gram-positive bacteria. However, the authors were able to generate an unconditional vicR insertional mutant of group A Streptococcus. This mutant grew well in rich media but not in non-immune human blood and serum, had attenuated virulence, and was unstable in mice. Complementation of the mutant with vicR expressed in trans restored its phenotype to wild-type. A vicK deletion mutant had a phenotype similar to that of the vicR mutant. Phagocytosis and killing of the vicR mutant were normal, suggesting that VicRK does not regulate processes involved in evasion of host defence. Microarray analysis showed that vicR inactivation down-regulated the transcription of 13 genes, including putative cell wall hydrolase gene pcsB and spy1058–1060, which encode a putative phosphotransferase system enzyme II for carbohydrate transport, and upregulated the expression of five genes, including spy0183 and spy0184, which encode an osmoprotectant transporter OpuA. Consistent with microarray analysis, the vicR mutant took up more of the osmoprotectants betaine and proline and was sensitive to osmotic stress, indicating that vicR inactivation induced osmotic stress and increased susceptibility to osmotic pressure. Additionally, a spy1060 deletion mutant also displayed attenuated virulence. These results suggest that VicRK regulates processes involved in cell wall metabolism, nutrient uptake, and osmotic protection.

INTRODUCTION

Streptococcus pyogenes or group A Streptococcus (GAS) is a major human pathogen (Cunningham, 2000). This Gram-positive organism causes a variety of superficial infections, including pharyngitis and impetigo. In addition, pharyngitis patients may develop scarlet fever and post-infection sequelae, such as acute rheumatic fever and glomerulonephritis. If GAS crosses the skin barrier, it can cause lethal necrotizing fasciitis, bacteraemia, and streptococcal toxic shock syndrome. Whereas streptococcal pharyngitis can be treated effectively with antibiotics, severe invasive infections usually progress rapidly, are difficult to treat (Kaul et al., 1999; Stevens, 1995), and can have mortality rates of up to 85% (Adams et al., 1985).

A thorough understanding of GAS virulence factors is essential in developing novel strategies to combat infections caused by this organism. The expression of many virulence factors is regulated by environmental signals (Caparon et al., 1992; Mekalanos, 1992), some of which are transduced by specific two-component regulatory systems (TCRs) (Hoch, 2000). TCRs consist of membrane protein sensors and cognate cytoplasmic response regulators. The sensor undergoes autophosphorylation on a histidine residue in response to a specific environmental signal and relays the phosphate group to an aspartic acid residue of the regulator. The phosphorylated regulator then binds to target DNA elements with greater affinity, activating or repressing the transcription of target genes (Rammersaud et al., 1994). GAS has 13 known/putative TCRs (Graham et al., 2002). CovR/CovS or CsrR/CsrS responds to Mg\(^{2+}\) concentration (Gryllos et al., 2003) and down-regulates the expression of several known virulence factors (Levin & Wessels, 1998; Federle et al., 1999; Heath et al., 1999). Another TCR, Ihk-Irr, senses an unknown signal from primary granules of human polymorphonuclear leukocytes (PMNs), and attenuates virulence in mice (Voyich et al., 2004). FasBCA is involved in the regulation of several growth-phase-associated virulence factors (Kreikemeyer...
et al., 2001). The regulons of the other ten putative TCRs are not known, although it is known that nine of them, excluding Spy0528/Spy0529, do not have targets that overlap with that of CovR/CovS (Ribardo et al., 2004).

Spy0528/Spy0529 is homologous to YycFG or VicRK (referred to VicR in this report). VicR is essential in Bacillus subtilis (Fabret & Hoch, 1998), Staphylococcus aureus (Martin et al., 1999) and Streptococcus pneumoniae (Lange et al., 1999; Throup et al., 2000), and vicR cannot be inactivated unless VicR is provided in trans to these organisms (Throup et al., 2000) or PcsB is constitutively expressed in S. pneumoniae (Ng et al., 2003). VicK is also essential in B. subtilis, but not in S. pneumoniae and Streptococcus mutans (Fabret & Hoch, 1998; Echenique & Trombe, 2001; Senadheera et al., 2005). Since an unconditional vicR mutant is not available, various strategies, including bioinformatic analysis, construction of hybrid regulator, depletion and overexpression of VicRK, and vicK inactivation, have been used to identify putative targets of VicRK (Fukuchi et al., 2000; Howell et al., 2003; Ng et al., 2003; Durac & Msadek, 2004; Mohedano et al., 2005; Senadheera et al., 2005). Nevertheless, the essential processes regulated by VicRK in these organisms remain unknown. Defects in morphology and cell wall synthesis, decreased competence, sensitivity to antibiotics and fatty acids, attenuated virulence, and defects in biofilm formation have been associated with null vicR mutants or conditional mutants under non-functional conditions for VicR (Martin et al., 1999; Echenique & Trombe, 2001; Kadioglu et al., 2003; Ng et al., 2004; Senadheera et al., 2005). Some of these phenotypes appear to be organism-specific; however, no studies on GAS VicR have been reported. Whether VicR is essential and what phenotypes are associated with defective VicR mutants are not known in GAS.

In the present study, we report the generation of a novel mutant of the vicR gene in GAS, and demonstrate that it exhibits unique phenotypes for growth and sensitivity to osmotic stress. In addition, microarray analysis was used to identify genes differentially regulated as a result of vicR inactivation. Our results suggest that VicRK is involved in regulation of cell wall metabolism, nutrient uptake, and osmotic protection.

**METHODS**

**GAS strains and growth.** Serotype M1 GAS strain MGAS5005 has been described previously (Hoe et al., 1999). MGAS5005 and its mutant strains were routinely grown at 37 °C in 5% CO2 in Todd–Hewitt broth supplemented with 0.2% yeast extract (THY) and appropriate antibiotics (150 mg spectinomycin l−1 and/or 25 mg chloramphenicol l−1) for the mutants and their derivative strains.

**Construction of vicR and spy0527 insertional and vicK and spy1060 deletion mutants.** An MGAS5005 mutant strain defective in vicR (vicR::aad) was generated by insertion mutagenesis. The vector pFWaad, derived from the streptococcal gene-inactivation vector pFW14 (Podbielski et al., 1996) by replacing its cat gene with the spectinomycin-resistance gene aad (LeBlanc et al., 1991), has been described previously (Hanks et al., 2005). An internal 403 bp fragment (bases 19–421) of the vicR gene was PCR-amplified using primers vicRIP1 and vicRP2 (Table 1). The PCR product was cloned into pFWaad at the EcoRI and Ncol sites to yield suicide plasmid pFWaad-vicR. Restriction-enzyme digestion showed that pFWaad-vicR lacked the Ncol site, and DNA sequencing showed that loss of the Ncol site was due to loss of the Ncol-containing 27 bp sequence between the EcoRI site and the sequence AGITCC of pFWaad. As a result, there was a sequence of TAGCA between the stop codon of aad and the 5′ end of the 3′ fragment of interrupted vicR. The suicide plasmid was introduced into MGAS5005 by electroporation, and vicR::aad mutants were selected with spectinomycin.

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**Table 1. Primers used for gene inactivation and complementation**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Amplicon</th>
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<tbody>
<tr>
<td>vicRIP1</td>
<td>ACCATGGATATGAAAAACCGATTTC</td>
<td>vicR internal fragment for insertional mutagenesis</td>
</tr>
<tr>
<td>vicRP1</td>
<td>CGAATTCAATGTCATTTGCTGTAC</td>
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</tr>
<tr>
<td>527IP1</td>
<td>ACCATGCTAGAGATCGACATTGACG</td>
<td>spy0527 internal fragment for insertional mutagenesis</td>
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<td>527IP2</td>
<td>ACCATGGCAGGCGCTTGCC</td>
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<tr>
<td>aadP1</td>
<td>GAAGATCTGATACATGTTAATAAATCTATAAC</td>
<td>aad to obtain pGRV</td>
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<tr>
<td>aadP2</td>
<td>CCGGATCCGGGCATGTGATTTTCTCC</td>
<td></td>
</tr>
<tr>
<td>vicKFIP1</td>
<td>TATGGAAGCTTGAATATCATTGGAATTATCC</td>
<td>The upstream fragment of vicK for vicK deletion</td>
</tr>
<tr>
<td>vicKFIP2</td>
<td>GGATCTTCTGTGCTGTGTGGT</td>
<td>The downstream fragment of vicK for vicK deletion</td>
</tr>
<tr>
<td>vicKFIP2</td>
<td>CGGGATCAAGGTGACCCATCATGAAG</td>
<td></td>
</tr>
<tr>
<td>1060FIP1</td>
<td>TATGGCGAGCTAAGCTGACACGTAACCATC</td>
<td>The upstream fragment of spy1060 for spy1060 deletion</td>
</tr>
<tr>
<td>1060FIP2</td>
<td>GGAGATCTAAAACGGAGGGAGTGTGAGAC</td>
<td>The downstream fragment of spy1060 for spy1060 deletion</td>
</tr>
<tr>
<td>vicRIP1</td>
<td>CGAGTCCAAATAAGAATTTCTGAGGATAACAG</td>
<td>vicR for complementation</td>
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<tr>
<td>vicRP2</td>
<td>AGGATTCTAGCTACAGATGTTGCGT</td>
<td>vicR for protein production</td>
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and confirmed by PCR and DNA sequence analyses. An insertional mutant of spy0527 (spy0527::aad), which is upstream of vicR, was similarly constructed.

To generate vicK and spy1060 deletion mutants (∆vicK and ∆spy1060), the aad gene was amplified using primers aadP1 and aadP2 and cloned into pWW14 at the BglII and BamHI sites to yield pGRV, the gene replacement vector. The upstream and downstream flanking fragments of the deleted internal fragment (bases 609–1309) of vicK were PCR-amplified using paired primers vicKFP1/vicKFP2 and vicKFP2/vicKFP2, respectively. The PCR products of the upstream and downstream fragments were sequentially cloned into pGRV at the HindIII/BglII and BamHI/SalI sites, respectively, to yield pGRV∆vicK. pGRRSpy1060 was similarly constructed using the flanking fragments of the deleted internal fragment (bases 154–685) of spy1060, which were PCR-amplified using paired primers 1060FP1/1060FP1 and 1060FP2/1060FP2. pGRV∆vicK and pGRRSpy1060 were both introduced into MGAS5005 by electroporation. The deletion mutants, which were resistant to spectinomycin and sensitive to chloramphenicol, were confirmed by PCR and DNA sequencing analyses.

**Plasmid pCMVvicR for complementation of vicR::aad.** A DNA fragment containing vicR and its ribosome-binding site was amplified from MGAS5005 using primers vicRP1 and vicRP2. The PCR product was cloned into pCMV (Hanks et al., 2005) at the BamHI site, yielding pCMVvicR. The cloned gene was sequenced to rule out spurious mutations and confirm the desired orientation.

**GAS growth in human blood.** Blood of healthy individuals was collected in accordance with a protocol approved by the Institutional Review Board at Montana State University, Bozeman. Non-immune donors were identified by the following criteria: 1) absence of GAS-specific antibodies in their sera, and 2) growth of GAS and non-survival of immune-serum-treated GAS in their blood. GAS strains were harvested at exponential growth phase, washed three times with pyrogen-free Dulbecco's phosphate-buffered saline (DPBS), and inoculated at ~10^8 c.f.u. ml^{-1} into heparinized non-immune blood. The samples were rotated end-to-end at 37 °C for 4 h, and numbers of viable GAS in the samples and actual inocula were determined by plating on THY agar. Growth factor was defined as the ratio of c.f.u. of each sample after 4 h incubation to c.f.u. in the inoculum.

**Growth-competition assays.** MGAS5005 and ∆vicK harvested from exponential growth phase were washed three times with DPBS, adjusted to ~10^8 c.f.u. ml^{-1} in DPBS, and mixed together in an approximate mutant : wild-type (wt) ratio. The bacterial mixture was inoculated into 1 ml human blood (inoculum = 10^6 c.f.u. per mouse). The triplicate human blood cultures were incubated at 37 °C for 4 h, and mouse blood was collected from the mice at 10 h after inoculation and plated on blood agar. Mutant : wt ratios of colonies from the samples and the starting bacterial mixture were determined by testing 100 GAS colonies per sample for their resistance to spectinomycin.

**Mouse infection.** GAS strains were harvested at exponential phase, washed with DPBS, and inoculated subcutaneously at the indicated inoculum into groups of eight to ten five-week-old female outbred CD-1 Swiss mice (Charles River Laboratory). Survival rates were examined daily for 14 days after inoculation. To test the stability of insertional mutant strains in mice, 5 × 10^7 c.f.u. of bacteria was inoculated intraperitoneally into six CD-1 mice per strain, and blood samples were collected at 9 and 22 h after inoculation and plated on blood agar. All animal procedures were approved by the Institutional Animal Care and Use Committee at Montana State University, Bozeman.

**Stability of the vicR mutant.** Cultures of vicR::aad in THY without spectinomycin at the late-exponential growth phase were inoculated into fresh THY sequentially for up to six passages. Triplicates of thirty colonies randomly chosen from THY agar for each passage were tested for growth on THY agar plates with spectinomycin. The resistance test was performed in a similar manner for colonies from mouse blood collected at 9 and 22 h after intraperitoneal inoculation of mice with strains vicR::aad, vicR::aad pCMVvicR, and spy0527::aad (control).

**Phagocytosis and killing assays.** MGAS5005 and vicR mutant from the exponential growth phase in THY were washed with DPBS and labelled with 0-75 μg ml^{-1} FITC in DPBS at 37 °C for 20 min. The labelled bacteria were washed and resuspended at 1 × 10^8 c.f.u. ml^{-1} in DPBS. Phagocytosis was performed as described by White-Owen et al. (1992). Briefly, 10 μl of the labelled bacteria was mixed with 100 μl immune or non-immune heparinized human blood and incubated with gentle shaking at 37 °C for 0, 15, 30 or 60 min. The samples were immediately processed using an Immunolysel kit (Beckman Coulter) according to the manufacturer's protocol and analysed by flow cytometry. The percentage of PMNs with fluorescent bacteria was used as a measurement of phagocytosis efficiency. Bacterial killing by isolated PMNs was performed as described by Lei et al. (2001).

**TaqMan real-time RT-PCR.** TaqMan analysis to determine levels of pCSB, spy1060, spy0184, emm and vicK was performed in triplicate with total RNA of the wt, vicR::aad, vicR::aad pCMVvicR and ∆vicK using an ABI 7700 thermocycler and specific TaqMan primers and probes (AlleLogic Biosciences), as described by Lei et al. (2003).

**Microarray analysis.** Total RNA was isolated from GAS at exponential phase with Qiagen RNeasy kits, as described by Lei et al. (2003). Labelled cDNA was generated from GAS RNA and hybridized to Affymetrix NimbleExpress Streptococcus pyogenes arrays, which contained eight sense/antisense pairs of probes for each of 1705 M1 GAS ORFs, based on serotype M1 strain SF370 (Ferretti et al., 2001). GeneSpring software was used to identify genes with ≥ 2-fold change in transcript levels in vicR::aad compared with the wt and vicR::aad pCMVvicR strains, using the data normalized with per chip per gene median polishing.

**Recombinant proteins and antisera.** Mouse antisera against recombinant FtsB and VicR were obtained as described by Lei et al. (2000). FtsB was purified as described by Lei et al. (2004). To prepare recombinant VicR, the vicR gene was cloned from MGAS5005 using primers vicRP1 and vicRP2. The PCR product was digested with NcoI and EcoRI and ligated into PET21d at the NcoI and EcoRI sites to yield pVICR. Recombinant VicR produced from this plasmid was tagged free. The gene clone was sequenced to rule out the presence of spurious mutations.

VicR was expressed in Escherichia coli BL21 (DE3) containing pVICR. The bacteria were grown to OD_{600} ≈ 0.5 in 4 luria–Bertani broth containing 100 mg ampicillin l^{-1} at 37 °C, and expression was induced with 0-5 mM IPTG for 6 h. Cell pastes obtained were sonicated in 60 ml 20 mM Tris/HCl, pH 8.0, on ice for 15 min, and the sample was centrifuged at 20,000 g for 15 min. Since the majority of VicR was expressed in inclusion bodies, insoluble VicR was dissolved in 8 M urea in Tris/HCl and refolded by adding 40 vols 20 mM Tris/HCl to 1 vol. VicR/urea solution with gentle stirring. After 15 min, the sample was loaded onto a DEAE-Sepharose column (2.5 × 15 cm), and the column was eluted with 0-18 M NaCl in Tris/HCl, pH 8. Fractions containing VicR were pooled, precipitated with ammonium sulfate at 70% saturation, and centrifuged. The VicR pellet was dissolved in Tris/HCl and dialysed against 3 l Tris/HCl. The VicR obtained was more than 95% pure, as assessed by SDS-PAGE.

**Western blotting analysis.** VicR and FtsB (control) produced by GAS strains were detected by Western blotting using mouse anti-VicR and anti-FtsB antisera, respectively, as described by Lei et al. (2001).
(2000). To prepare samples for the analysis, bacteria were harvested from 40 ml cultures at OD_{600} 0.4, washed twice with 1.5 ml PBS, and treated with 200 U mutanolysin in 100 μl PBS at 37 °C for 2 h. The samples were briefly sonicated, and mixed with an equal volume of 2× SDS-PAGE loading buffer.

Scanning electron microscopy (SEM) analysis. The wt and vicR::aad bacteria harvested at exponential growth phase were pelleted with H_{2}O, spotted on wafers, and coated with gold/palladium. SEM images were taken at a magnification of 20,000 using a JEOL JSM6100 SEM microscope.

Uptake of radiolabelled chemicals. wt and vicR::aad bacteria from 35 ml cultures at exponential growth phase were pelleted by centrifugation and resuspended in 3·5 ml THY. For uptake at each time-point, triplicate samples were prepared by mixing 0·25 ml bacterial suspension with 0·25 ml THY containing 0·5 μCi (18·5 MBq) L-[2,3,4,5-3H]proline (104 Ci mmol⁻¹, 3848 GBq mmol⁻¹, Amersham Biosciences), 0·025 μCi (0·925 MBq) [methyl-14C]betaine (55 Ci mmol⁻¹, 2035 GBq mmol⁻¹, American Radiolabelled Chemicals, Inc.) or 0·5 μCi (18·5 MBq) D-[6-3H]glucose. The samples were rotated end-to-end at room temperature, pelleted at the desired time, and washed twice with 1 ml PBS. The bacterial pellets obtained were resuspended in 0·2 ml PBS and mixed with 3 ml scintillation cocktail. ^3H or ^14C radioactivity associated with bacteria was quantified using a Beckman LS 6500 scintillation counter.

RESULTS

Generation of a GAS vicR mutant

Introduction of a suicide plasmid containing an internal vicR fragment into MGAS5005 resulted in a total of three colonies in two independent experiments, and DNA sequence analysis indicated that the strains had the suicide plasmid inserted into the vicR gene. Notably, the frequency of obtaining the mutant (vicR::aad) was three orders of magnitude lower than that of obtaining an insertional mutant of spy0527 (spy0527::aad), the upstream gene of vicR. The production of VicR was detected in the parent strain but not in vicR::aad (Fig. 1), confirming that the insertion inactivated the vicR gene.

Fig. 1. Analysis of VicR production in GAS strains. Proteins from 2·7×10^9 cells of each strain were probed with mouse anti-VicR and anti-FtsB (control) antisera. Lanes: 1, recombinant VicR; 2, wt; 3, vicR::aad; 4, vicR::aad revertant; 5, vicR::aad/pCMVvicR; 6, ΔvicK. The results presented are representative of three experiments.

Growth of vicR::aad in THY, human blood, and serum

The vicR::aad mutant grown in THY did not show morphological defects, as determined by SEM, although the sizes of vicR::aad colonies on THY agar were about one-half those of the wt strain (data not shown). The shapes of vicR::aad growth curves in THY were similar to those of the wt strain under identical conditions; however, the early growth phase of vicR::aad was 30 and 180 min longer than that of the wt strain when the starting bacteria were from cultures at exponential and stationary phase, respectively (Fig. 2A). Values for c.f.u. per OD_{600} unit of the starting exponential and stationary vicR::aad cultures were 45 and 24% of those of wt cultures, respectively (Fig. 2B), suggesting that a smaller number of viable vicR::aad bacteria was the reason for the longer early growth phase of vicR::aad. These results indicate that vicR::aad grew as well as the wt.

Fig. 2. Growth and viability of vicR::aad in THY. (A) Growth curves of the vicR::aad (squares) and wt (circles) strains in THY. Cultures at the exponential phase (solid symbols) or 20 h after reaching OD_{600} 0·75 (open symbols) were inoculated into fresh THY, and OD_{600} was measured at the indicated times. For culture of the mutant, THY was supplemented with spectinomycin. (B) Viability of vicR::aad (solid bars) and wt (open bars) cultures in THY at exponential (Exp), early stationary (reaching OD_{600} 0·75, E St), and late-stationary (20 h after reaching OD_{600} 0·75, L St) growth phases. The numbers above the solid bars are vicR::aad/wt viability ratios at the corresponding growth phases. The results presented are representative of three experiments.
strain in THY, but that the mutant had lower viability, especially in stationary culture.

The wt strain grew rapidly in non-immune human blood, with a growth factor (see Methods) in four independent experiments of 320–1080 after incubation at 37 °C for 4 h. In contrast, the vicR mutant had a smaller growth factor of 5–38. The results of one experiment are shown in Fig. 3. Although the growth factors of the strains were also significantly different in THY (P = 0.0051), the growth factor ratio of wt to vicR::aad in THY was only 3, which was dramatically less than 74, the wt to vicR::aad growth factor ratio in blood (Fig. 3). Furthermore, vicR::aad (growth factor = 38) did not grow as well as the parent strain (growth factor = 1005) in serum obtained from the same individual (P = 0.0001) (Fig. 3), suggesting that the smaller growth factor of vicR::aad in human blood was primarily due to a growth defect.

To determine whether the slower growth of vicR::aad in blood was due to more efficient killing of the mutant by PMNs, the phagocytosis of wt and vicR::aad bacteria by PMNs in immune and non-immune blood was examined. Percentages of PMNs in immune blood with phagocytosed wt or vicR::aad were significantly higher than those in non-immune blood at the time-points of 15 and 30 min (P < 0.05) (Fig. 4), consistent with the ability of protective GAS-specific antibodies to enhance phagocytosis by PMNs. There was no significant difference in phagocytosis of wt and vicR::aad strains between non-immune or immune blood (P > 0.05) (Fig. 4). The lower phagocytic efficiency in non-immune blood was due to a PMN defect, since GAS treated with immune serum did not survive in the non-immune blood (data not shown). These results indicate that, like wt GAS, vicR::aad still possessed an anti-phagocytic phenotype. Consistent with these results, there was no significant difference in killing of wt and vicR::aad bacteria by isolated PMNs (data not shown). To further distinguish between a growth defect and phagocytic resistance, the growth of the wt and vicR::aad strains in blood was compared in both rotated and stationary tubes. If the defect of vicR::aad in growth in blood is indeed independent of phagocytosis, the poor growth of the mutant should persist under stationary conditions. The wt strain grew well under both conditions, with similar growth factors, while vicR::aad had a similar small growth factor under both rotated and stationary conditions, confirming that the poor growth of vicR::aad in blood was not due to loss of resistance to phagocytosis. Thus, GAS killing by PMNs did not contribute to the difference in the ability of the wt and vicR::aad strains to grow in blood.

**Restoration of wt phenotype by reversion and complementation**

Excision of the suicide plasmid in the absence of spectinomycin generated several spectinomycin-sensitive revertants of vicR::aad. As expected, the revertants had an intact vicR gene, based on DNA sequencing. One revertant was randomly chosen for further tests. The revertant restored production of VicR (Fig. 1) and, more importantly, restored the ability to grow in human blood and serum (Fig. 3). In addition, complementation of vicR::aad with the vicR gene expressed in trans from plasmid pCMV vicR restored the production of VicR (Fig. 1) and the ability of the strain to grow in human blood and serum (Fig. 3). Thus, these data clearly demonstrate that the defective growth phenotype of vicR::aad was
due to the absence of VicR, and was not due to a possible secondary mutation or polar effect.

**Attenuation of GAS virulence by vicR inactivation**

Groups of mice were subcutaneously infected with wt, vicR::aad, vicR::aad revertant, vicR::aad/pCMVvicR, and vicR::aad/pCMV (vector control). The wt and vicR::aad strains were tested in one experiment using ten mice in each group, and then tested together with the other strains in a second experiment using eight mice per group strain. Seventeen of the 18 mice infected with wt GAS died, while 13 of the 18 mice infected with vicR::aad survived (Fig. 5), indicating that vicR inactivation significantly attenuated GAS virulence (Logrank test: P<0.0001). Complementation of vicR::aad with pCMVvicR, but not the vector control strain, or reversion of vicR::aad, restored virulence (vicR::aad/pCMVvicR or vicR::aad revertant versus wt in the second experiment: P≥0.7343) (Fig. 5), indicating that the attenuation of virulence in vicR::aad was also associated with the lack of VicR.

**Instability of vicR::aad in mice**

No loss of the insertion in vicR::aad was detected within six passages in THY in the absence of spectinomycin. However, ~58 and 100% of GAS colonies derived from mouse blood lost resistance to spectinomycin at 9 and 22 h after intraperitoneal inoculation of vicR::aad, respectively (Table 2), and all three of the spectinomycin-sensitive colonies randomly chosen were in-frame reverants. In contrast, spy0527::aad was stable in the intraperitoneal mouse infection. Furthermore, all colonies tested from mouse blood at 9 and 22 h after intraperitoneal inoculation with vicR::aad/pCMVvicR were resistant to both spectinomycin and chloramphenicol, indicating that expression of the vicR gene in trans prevented vicR::aad from losing the insert. These results indicate that the vicR interruption was deleterious and that VicR was required for GAS survival in vivo.

**Genes potentially regulated by VicRK**

To identify potential targets of VicRK, transcription profiling was compared between wt, vicR::aad and vicR::aad/pCMVvicR strains using custom Affymetrix NimbleExpress *Streptococcus pyogenes* arrays. Microarray analysis was performed once for the wt versus vicR::aad comparison, and twice for the wt, vicR::aad, and vicR::aad/pCMVvicR comparison, each on different days. Transcript levels of 13 genes were down-regulated, while those of five other genes were upregulated by >1.5-fold in vicR::aad compared with both the wt and vicR::aad/pCMVvicR strains (Table 3). The small number of genes identified was due to the stringent requirement that any transcriptional changes induced

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**Table 2. Stability of vicR::aad in THY and mice**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Percentage of colonies resistant to spectinomycin (mean ± SD)*</th>
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<td></td>
<td>THY (six passages)</td>
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<td></td>
<td></td>
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<tr>
<td>vicR::aad</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>vicR::aad/pCMVvicR</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>spy0527::aad (control)</td>
<td>100 ± 0</td>
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*Resistance to spectinomycin was determined for colonies from exponential cultures of the strains after growing in THY without spectinomycin for six passages (three independent experiments) or from mouse blood at 9 and 22 h after intraperitoneal infection with ~5 × 10⁷ c.f.u. bacteria. For each strain, the mouse data were derived from at least three mice for each time-point.
by vicR inactivation were at least partially reversed in the complemented strain; the genes reported in Table 3 all met this requirement.

The genes with altered transcription included spy0019, a cluster of spy1057–1063, and spy0183/0184. The spy0019 gene encodes a putative murein hydrolase, whose homologues in *S. pneumoniae* and *S. mutans* are known to be positively regulated by VicRK (Ng et al., 2004; Durac & Msadek, 2004; Mohedano et al., 2005). The spy1057–1063 locus encodes a putative phosphotransferase system (PTS) enzyme II carbohydrate transporter (Spy1058–1060), a putative TCS (Spy1061/1062), a putative secreted iron-binding protein (Spy1063), and a hypothetical protein (Spy1057). The spy0183/0184 genes upregulated in vicR::aad encode a homologue of *Lactococcus lactis* osmoprotectant transporter OpuA. Thus, these results suggest that vicR inactivation may decrease nutrient uptake and introduce osmotic stress. Furthermore, inactivation of vicR also down-regulated transcription of genes encoding Mac (Lei et al., 2001), a collagen-like protein (Lukomski et al., 2000), and two genes (spy1181 and spy1183) of a locus encoding a putative citric acid lyase. The spy0145 and spy0146 genes, encoding a hypothetical protein and a putative regulatory protein, respectively, were also upregulated.

TaqMan analysis confirmed the microarray results for selected genes, spy0019, spy0184, and spy1060, as well as the control gene *emm* (Fig. 6). The *emm* gene, which encodes the dominant cell-surface M protein, was expressed at similar levels in all the strains.

In the vicR mutant, there was no transcription termination sequence between the *aad* gene, which included its native promoter (LeBlanc et al., 1991), and the 3′ fragment of the interrupted vicR gene. The transcript levels of *vicK* and *vicX* in vicR::aad were down-regulated compared with the wt strain (Fig. 6, Table 3), indicating a polar effect of the insertion and suggesting that the *vicRKX* genes are in an operon. However, this polar effect was not responsible for the phenotype of vicR::aad, since complementation of vicR::aad with pCMVvicR did not restore the level of *vicK* transcripts (Fig. 6), but did confer stability to the mutant in mice (Table 2), and also restored wt phenotypes.

### Table 3. Genes with altered transcription in the vicR::aad mutant compared with the wt and complemented vicR::aad/pCMVvicR strains

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<thead>
<tr>
<th>Gene</th>
<th>Relative fold-change</th>
<th>Known or putative protein</th>
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<td>Versus complemented strain</td>
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</tr>
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*The number in parentheses is the fold-change determined by TaqMan analysis. Negative and positive values indicate down- and upregulation, respectively, of gene transcription in vicR::aad compared with the wt or complemented mutant strains.

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for growth in non-immune blood (Fig. 3) and virulence (Fig. 5).

**Osmotic stress sensitivity and enhanced OpuA activity of vicR::aad**

Osmotic stress upregulates opuA transcription and activates betaine and proline uptake activities of OpuA in L. lactis (Obis et al., 1999; van der Heide & Poolman, 2000). Since transcription of opuA was upregulated in vicR::aad (Table 3), the vicR::aad mutant should have higher OpuA activity. To test this idea, the uptake of radioactively labelled betaine and proline by vicR::aad and wt strains was examined. Indeed, the rates of [14C]betaine and [3H]proline uptake by the mutant were 190 and 40 % higher, respectively, than those of the wt strain (Fig. 7), using amounts of bacteria based on their OD600. These levels of enhancement are conservative estimates, however, since vicR::aad had lower viability per OD600 than the wt strain. In any case, these results suggest that OpuA activity was enhanced in vicR::aad, implying that the vicR inactivation introduced osmotic stress. Moreover, vicR::aad grew much more slowly in THY with additional (0-3 M) NaCl than in THY, while there was no dramatic difference in growth of the wt strain in THY with or without the added 0-3 M NaCl (Fig. 7C), providing further evidence that vicR::aad was sensitive to osmotic stress.

**Similar phenotypes of ΔvicK and vicR::aad mutants**

Since vicR::aad was not stable in mice, a stable vicR deletion mutant was desirable. However, several attempts to obtain vicR deletion mutants were unsuccessful. Considering that inactivation of vicK should at least partially block the function of VicRK, we decided to generate vicK deletion mutants (ΔvicK) as an alternative solution. ΔvicK mutants were easily obtained through allelic exchange, suggesting that there was no selection pressure against ΔvicK construction. The vicK deletion did not affect the expression of VicR (Fig. 1). The vicK deletion decreased the levels of pcsB and spy1060 transcripts, and upregulated spy0184 transcription, but to a lesser extent than with vicR inactivation (Fig. 6). The growth factor of ΔvicK in non-immune human blood
was 13% of that of the wt strain. To assess the growth of ΔvicK in mice in comparison with the wt strain, a mixture of bacteria with a ΔvicK: wt ratio of 2:7 was inoculated into human blood or intraperitoneally into mice. After 4 h incubation in human blood and at 10 h after infection, the ΔvicK: wt ratios of GAS colonies from the human blood culture and blood of infected mice were 0.41 and 0.01, respectively. Thus, these results suggest that ΔvicK also had a growth defect in human blood and in mice, and could not compete against the wt strain in human blood and in mice. Consistent with the growth-competition result, ΔvicK did not kill mice under the conditions in which all of the mice infected with the wt strain died (Fig. 8). Thus, ΔvicK and vicR::aad had similar phenotypes for ex vivo and in vivo growth and virulence.

**Attenuation of GAS virulence by spy1060 deletion**

To examine whether the down-regulation of spy1058–spy1060 transcription contributed to the phenotype of vicR::aad, a deletion mutant of spy1060 (Δspy1060) was generated by allelic exchange, and its growth in human blood and virulence in mice were examined. The growth factor of Δspy1060 was 66% of that of the wt strain, and the virulence of Δspy1060 was significantly attenuated in a mouse model of subcutaneous infection (Δspy1060 versus wt: P = 0.0048) (Fig. 8). Thus, these results suggested that down-regulation of spy1058–1060 transcription contributed to the phenotype of vicR::aad. To determine whether Spy1058–1060 takes up glucose, the uptake of [3H]D-glucose was monitored in the wt, vicR::aad, and Δspy1060. There was no significant difference in [3H]D-glucose uptake among the strains (data not shown), suggesting that Spy1058–1060 is not a major transporter for glucose.

**DISCUSSION**

In the present study, we describe the generation and characterization of an insertional mutant of vicR in a serotype M1 GAS strain, which is the first unconditional vicR mutant reported in Gram-positive bacteria. The mutation was confirmed by PCR, DNA sequencing, and Western blotting analyses. Although an earlier attempt to inactivate the GAS vicR gene was unsuccessful (Federle et al., 1999), we suggest that this outcome was possibly due to the extremely low frequency of obtaining the vicR::aad mutant, and that the large-scale electroporation used here could be an important factor in generating such rare mutants. Alternatively, it may have been the case that one of the truncated alleles of vicR in our vicR insertion mutant was expressed, albeit at a level undetectable by Western blot, and had partial activity, and that this allowed us to obtain the mutant. Although the generation of the vicR::aad mutant suggests that VicR may not be essential, the mutant was not stable in vivo. Furthermore, we were unable to construct a vicR deletion mutant. Thus, we were unable to conclude whether VicR is essential in GAS.

The vicR gene is essential in B. subtilis, Staph. aureus and S. pneumoniae (Fabret & Hoch, 1998; Martin et al., 1999; Lange et al., 1999; Throup et al., 2000), and can be inactivated in Staph. aureus and S. pneumoniae only when vicR is expressed in trans (Fabret & Hoch, 1998; Throup et al., 2000). In addition, constitutive in trans expression of pcsB also allows the inactivation of S. pneumoniae vicR (Ng et al., 2003). Based on these observations in other bacteria, we considered several possible explanations for the successful generation of a GAS vicR::aad mutant. As mentioned above, a truncated VicR with partial function might be produced in the mutant. Another possibility is that VicRK might not be essential in GAS. Only one of the putative targets of VicRK in GAS that we identified, pcsB (spy0019), had a homologue in the putative targets of VicRK identified in other bacteria (Howell et al., 2003; Ng et al., 2003; Durac & Msadek, 2004; Mohedano et al., 2005; Senadheera et al., 2005), suggesting that VicRK may regulate distinct subsets of genes in different bacteria. Thus, it is plausible that the essential genes regulated by VicRK in B. subtilis, Staph. aureus and S. pneumoniae are not regulated by VicRK in GAS. A third possibility is that PcsB may not be essential in GAS. PcsB is essential in S. pneumoniae (Ng et al., 2004) and Enterococcus faecium (Teng et al., 2003), but not in Streptococcus agalactiae, S. mutans or Streptococcus thermophilus (Reinscheid et al., 2001; Chia et al., 2001; Borges et al., 2005). Thus, GAS PcsB may not be essential. A fourth possibility is that the basal expression of PcsB in the absence of VicR was enough for the survival of the GAS vicR::aad mutant, if PcsB is essential in GAS. A fifth possibility is that the differences among organisms in tolerance to osmotic stress could play a critical role. GAS grew normally in THY plus 0.3 M NaCl (Fig. 7C), but the growth of S. pneumoniae in THY plus 0.1 M NaCl is dramatically decreased compared with its growth in THY (Brown et al., 2004). We showed that vicR inactivation introduced osmotic stress and enhanced
susceptibility to osmotic stress. It is possible that the S. pneumoniae vicR mutant could not survive osmotic stress and enhanced sensitivity to osmotic stress, which might have resulted from vicR inactivation in S. pneumoniae. A final possibility considered is that a secondary mutation might be selected during the generation of the vicR::aad mutant. Although we could not rule out a secondary mutant in vicR, as discussed below, the observed phenotype of vicR::aad was not due to a possible secondary mutation but due to the lack of VicR.

The vicR::aad mutant possessed the following novel phenotypes: 1) while the vicR mutant grew well in THY and lacked defects in morphology, it did not grow well in non-immune human blood and serum; 2) it exhibited attenuated virulence in mice; 3) vicR inactivation was deleterious to GAS survival in mice; and 4) it was more sensitive to osmotic stress than the wt strain. All GAS colonies recovered from mouse blood at 22 h after intraperitoneal injection of vicR::aad were revertants. This was not due to a high frequency of reversion of the mutant, since no reversion was detected in THY without spectinomycin for up to six passages. Thus, it appears that a few revertants rapidly outgrew the mutant. These phenotypes were due to the lack of VicR, and not due to a polar effect or a potential secondary mutation, as demonstrated by complementation and reversion of vicR::aad. ΔvicK could be generated at a frequency comparable to that of obtaining Δspy1060, suggesting that there was no selection pressure for a secondary mutation during the generation of ΔvicK; however, both ΔvicK and vicR::aad mutants showed similar phenotypes, further supporting the idea that the phenotype of vicR::aad was due to vicR inactivation. Thus, we conclude that both VicR and VicK are critical for GAS growth in human blood and survival in mice. The difference in the ability of vicR::aad to grow under the different conditions and its sensitivity to osmotic stress have not been shown to be associated with VicRK in other Gram-positive organisms, and thus they are either unique phenotypes in GAS or newly described ones associated with VicRK.

The vicR mutant maintained anti-phagocytic activity and was not efficiently killed by isolated human PMNs, consistent with TaqMan data that showed that the transcript levels of emm, which encodes a major anti-phagocytic determinant, were similar in the wt and vicR::aad strains. These results suggest that the processes regulated by VicRK are not involved in evasion of host defences, but in bacterial growth. B. subtilis, Staph. aureus, and S. pneumoniae vicR mutants stop growing at non-permissive temperatures or when VicR expressed in trans is depleted (Fabret & Hoch, 1998; Martin et al., 1999; Ng et al., 2004). The reasons for the inability of these mutants to grow under such conditions may be similar to those leading to the inability of the GAS vicR mutant to grow in human blood and survive in mice.

VicK is essential in B. subtilis, but not in S. pneumoniae and S. mutans. We show here that VicK is not essential in GAS. The vicK deletion had effects, except for virulence, which were similar to but less dramatic than those caused by vicR inactivation, including the effects on the transcription of pcsB, spy1060 and spy0184, and on growth in human blood. These effects correlated well with the relative ease of obtaining vicK mutants. These results suggest that VicR could be activated by other TCR kinases or small-molecule phosphodinors. The latter could be more important than the former in ΔvicK, since the histidine kinases generally function to control the rate of both phosphorylation and dephosphorylation of the regulators, and a lowered dephosphorylation rate of phosphorylated VicR would allow the accumulation of VicR phosphorylated with phosphate groups from small phosphodinors. vicR::aad was not stable in mice, and the death of a few mice infected with vicR::aad might be caused by reverted wt bacteria. This possibility was supported by the fact that the stable ΔvicK mutant was more attenuated in virulence than vicR::aad. The transcription levels of pcsB, spy0184 and spy1060 in ΔvicK were very similar to those in vicR::aad/pCMVvicR in THY (Fig. 6). In contrast, vicR::aad/pCMVvicR was virulent, while ΔvicK was not, suggesting that the dependence of the transcription of these genes on VicRK may be more stringent in vivo.

Gene transcription profiling analysis identified three loci, which are or may be relevant to the phenotype of the vicR mutant. Transcription of pcsB is positively regulated by VicRK in S. pneumoniae and S. mutans (Ng et al., 2004; Durac & Msadek, 2004; Mohedano et al., 2005). The transcription of pcsB was down-regulated in vicR::aad, suggesting that VicRK also regulates pcsB expression in GAS. Thus, these findings suggest that the pcsB gene could be a common target of VicRK in Gram-positive bacteria. Constitutive expression of pcsB turns essential VicR into a non-essential factor in S. pneumoniae, suggesting that the essential requirement of VicRK in S. pneumoniae is due to the positive regulation of PcsB expression (Ng et al., 2003). However, the lack of an unconditional S. pneumoniae vicR mutant made it difficult to test this hypothesis. The availability of the GAS vicR::aad mutant reported here would provide an excellent opportunity to examine this issue, and studies to determine whether down-regulation of pcsB transcription in vicR::aad was a key factor responsible for its phenotype are in progress.

Down-regulation of the expression of SPy1058–1060 in vicR::aad could have contributed to its defective growth phenotype, and attenuation of virulence by spy1060 deletion supports this idea. Human blood is rich in carbohydrates and relatively poor in free amino acids (Goldman & Bennet, 2000). Carbohydrates appear to be the main energy source for GAS in human blood (Graham et al., 2005). GAS has at least four PTS carbohydrate transport systems, and SPy1058–1060 could be an important carbohydrate transporter in human blood and in vivo. We are currently determining the specificity of SPy1058–1060, which may provide information on a major energy source other than glucose for in vivo GAS growth. Our demonstration that
VicRK targets SPy1058–1060 also suggests that nutrient uptake could be one of the processes regulated by VicRK. Interestingly, the transcription of the spy1061 and 1062 genes, which are next to spy1058–1060 and encode a putative two-component system, was also down-regulated in vicR::aad. Thus, it is possible that VicRK and SPy1061/1062 function together to control preferences of nutrient utilization.

SPy0183 and SPy0184 share 67 and 58% identity in amino acid sequence with OpuAA and OpuABC, respectively, of the L. lactis osmoprotectant transporter OpuA. In L. lactis, osmotic stress both upregulates OpuA expression and activates OpuA, which transports the osmoprotectants betaine and proline (Obis et al., 1999; van der Heide & Poolman, 2000). Similarly, the upregulation of opuA transcription and enhanced rates of betaine and proline uptake in vicR::aad suggest that vicR inactivation resulted in osmotic stress. Indeed, vicR::aad was more sensitive to osmotic stress. Thus, the processes regulated by VicRK may also be important for cell wall integrity and osmotic protection.

Except for the common target pscB, to the best of our knowledge, the other genes with altered transcription in the vicR mutant have not been identified before as targets of VicRK in other Gram-positive bacteria (Ng et al., 2003; Mohedano et al., 2005; Durac & Msadek, 2004; Fukuchi et al., 2000; Howell et al., 2003). These data suggest that VicRK may regulate distinct subsets of genes in different bacteria. In addition, some of the previous studies (Ng et al., 2003; Durac & Msadek, 2004) used carbohydrate-inducible systems to achieve expression of VicRK, leading to the possibility that the use of carbohydrate or carbohydrate derivatives might have prevented identification of the PTS system as a possible target of VicRK.

In summary, we have generated a novel vicR mutant strain in GAS. We demonstrated that the vicR gene was critical for the growth and survival of GAS in human blood and in mice. We identified putative targets of VicRK in GAS and presented evidence suggesting that defects in nutrient uptake and the sensitivity to osmotic stress of the mutant may contribute to its phenotype. Further characterization of the putative targets of VicRK may unveil processes regulated by VicRK that are important for GAS survival in vivo, and also may lead to identification of novel therapeutic targets in GAS.

ACKNOWLEDGEMENTS

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