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.

Received 21 November 2005

Revised 22 December 2005

Accepted 5 January 2006

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 (Ramersaud *et al.*, 1994). GAS has 13 known/putative TCRs (Graham *et al.*, 2002). CovR/CovS or CsrR/CsrS responds to Mg²⁺ 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

Abbreviations: GAS, group A *Streptococcus*; PMN, polymorphonuclear leukocyte; PTS, phosphotransferase system; SEM, scanning electron microscopy; TCR, two-component regulatory system; wt, wild-type.

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 as VicRK 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 bioinformatics, 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 *vicK* 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 VicRK have been reported. Whether VicR is essential and what phenotypes are associated with defective VicRK 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 *vicRIP2* (Table 1). The PCR product was cloned into pFWaad at the *EcoRI* and *NcoI* sites to yield suicide plasmid pFWaad-*vicR*. Restriction-enzyme digestion showed that pFWaad-*vicR* lacked the *NcoI* site, and DNA sequencing showed that loss of the *NcoI* site was due to loss of the *NcoI*-containing 27 bp sequence between the *EcoRI* site and the sequence AGTTCC 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

Table 1. Primers used for gene inactivation and complementation

Primer	Sequence (5'–3')	Amplicon
<i>vicRIP1</i>	ACCATGGATGATGAAAAACCGATTTC	<i>vicR</i> internal fragment for insertional mutagenesis
<i>vicRIP2</i>	CGAATTCAATGGTCAGTTCCTGTGTAC	
<i>527IP1</i>	ACCATGGTAGAGATGCCATTTGAGC	<i>spy0527</i> internal fragment for insertional mutagenesis
<i>527IP2</i>	ACCATGGCCAGAGCTTGGGCGTC	
<i>aadP1</i>	GAAGATCTGAATACATGTTATAATAACTATAAC	<i>aad</i> to obtain pGRV
<i>aadP2</i>	CGGGATCCGGGCATGTGATTTTCCTCC	
<i>vicKF1P1</i>	TATGGAAGCTTAGATATCATTGGAAACTTATCC	The upstream fragment of <i>vicK</i> for <i>vicK</i> deletion
<i>vicKF1P2</i>	GAAGATCTTCCTGTTCTGTGGTGTGCATG	
<i>vicKF2P1</i>	CGGGATCCAAGATGCAGCCATCTATGAAG	The downstream fragment of <i>vicK</i> for <i>vicK</i> deletion
<i>vicKF2P2</i>	AGTGTCGACCGTAAAGTCTGTACCTACTG	
<i>1060F1P1</i>	TATGGAAGCTTAAGCTGCAGCAGCTAACCATC	The upstream fragment of <i>spy1060</i> for <i>spy1060</i> deletion
<i>1060F1P2</i>	GAAGATCTAAACGATTGATGACTGGTAG	
<i>1060F2P1</i>	CGGGATCCTTGACCAATTGTTTATTG	The downstream fragment of <i>spy1060</i> for <i>spy1060</i> deletion
<i>1060F2P2</i>	AGTGTCGACGTTGGTTTTGATCTATCATTG	
<i>vicRP1</i>	CGGATCCAATAAAGAACCTTCTGAGGTATAGAC	<i>vicR</i> for complementation
<i>vicRP2</i>	AGGATCCTAGTCATAAGATTTCATGTAG	
<i>vicRPP1</i>	ACCATGGAATAATACTTATTGTGGATGATG	<i>vicR</i> for protein production
<i>vicRPP2</i>	CGAATTCTAGTCATAAGATTTCATGTAG	

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 ($\Delta vicK$ and $\Delta spy1060$), the *aad* gene was amplified using primers *aadP1* and *aadP2* and cloned into pFW14 at the *Bgl*II and *Bam*HI 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 *vicKF1P1/vicKF1P2* and *vicKF2P1/vicKF2P2*, respectively. The PCR products of the upstream and downstream fragments were sequentially cloned into pGRV at the *Hind*III/*Bgl*II and *Bam*HI/*Sal*I sites, respectively, to yield pGRV $\Delta vicK$. pGRV $\Delta spy1060$ was similarly constructed using the flanking fragments of the deleted internal fragment (bases 154–685) of *spy1060*, which were PCR-amplified using paired primers *1060F1P1/1060F1P2* and *1060F2P1/1060F2P2*. pGRV $\Delta vicK$ and pGRV $\Delta spy1060$ 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 pCMV*vicR* 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 *Bam*HI site, yielding pCMV*vicR*. 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 $\sim 10^5$ 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 $\Delta vicK$ harvested from exponential growth phase were washed three times with DPBS, adjusted to $\sim 10^6$ c.f.u. ml⁻¹ in DPBS, and mixed together in an approximately 3:1 mutant: wild-type (wt) ratio. The bacterial mixture was inoculated into 1 ml human blood (inoculum = 10^5 c.f.u.) or intraperitoneally into three mice (inoculum = 5×10^7 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 or 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 1×10^9 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 Immunolyse 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 $\Delta vicK$ 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 $\geq$ or $\leq$ 1.5-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 *vicRPP1* and *vicRPP2*. The PCR product was digested with *Nco*I and *Eco*RI and ligated into pET21d at the *Nco*I and *Eco*RI sites to yield pVICR. Recombinant VicR produced from this plasmid was tag 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 OD₆₀₀ ~ 0.5 in 4 l 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.*

(2000). To prepare samples for the analysis, bacteria were harvested from 40 ml cultures at OD₆₀₀ 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 washed with H₂O, 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 µCi 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 2.7×10^8 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, $\Delta vicK$. 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 OD₆₀₀ 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 OD₆₀₀ 0.75 (open symbols) were inoculated into fresh THY, and OD₆₀₀ 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 OD₆₀₀ 0.75, E St), and late-stationary (20 h after reaching OD₆₀₀ 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 not 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

Fig. 3. Growth of GAS strains in non-immune human blood, serum, and THY. Approximately 10^5 c.f.u. of each strain was inoculated into 1 ml blood (solid bars), serum (open bars) or THY (hatched bars), in triplicate. c.f.u. of inocula and viable GAS in the samples after end-to-end rotation at 37 °C for 4 h were determined by plating. The growth factor (viable c.f.u./inoculum c.f.u.) $\pm$ SD of a representative experiment is presented. Strains: 1, wt; 2, *vicR::aad/pCMVvicR*; 3, *vicR::aad* revertant; 4, *vicR::aad*; 5, *vicR::aad/pCMV* (vector control).

Fig. 4. Phagocytosis of wt and *vicR::aad* bacteria by human PMNs. FITC-labelled bacteria (10^7 c.f.u.) were incubated with 100 μ l immune (solid bars) or non-immune (grey bars) heparinized human blood at 37 °C for 0, 15, 30 or 60 min. Red blood cells were lysed using an Immunolyse kit, and percentages of PMNs with phagocytosed bacteria determined by flow cytometry are presented. **Statistically significant difference ($P<0.05$) in phagocytosis between non-immune and immune blood samples.

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

Fig. 5. Attenuation of GAS virulence by *vicR* inactivation. Groups of female CD-1 mice were inoculated subcutaneously with the test strains, and survival rates were determined daily. The results for the wt and *vicR::aad* strains are the combined data from two independent experiments (one with ten mice per group and another with eight mice per group), and the data for the other strains were from the eight mice per group experiment. Inocula in c.f.u.: wt, 9.3×10^7 ; *vicR::aad*, 1.4×10^8 ; *vicR::aad* revertant, 1.1×10^8 ; *vicR::aad/pCMVvicR*, 1.1×10^8 ; *vicR::aad/pCMV*, 1.6×10^8 .

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 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 pCMV*vicR*, 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: $P \geq 0.7343$) (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 revertants. 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

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

Strain	Percentage of colonies resistant to spectinomycin (mean $\pm$ SD)*		
	THY (six passages)	Mice at time:	
		9 h	22 h
<i>vicR::aad</i>	100 $\pm$ 0	58 $\pm$ 21	0 $\pm$ 0
<i>vicR::aad/pCMVvicR</i>	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0
<i>spy0527::aad</i> (control)	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0

*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 $\sim 5 \times 10^7$ c.f.u. bacteria. For each strain, the mouse data were derived from at least three mice for each time-point.

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

Gene	Relative fold-change		Known or putative protein
	Versus wt	Versus complemented strain	
<i>spy0019</i>	-3.1 (-6.9)*	-2.6 (-3.7)	PcsB homologue
<i>spy0145</i>	3.1	2.3	Hypothetical protein
<i>spy0146</i>	3.6	2.3	Putative regulatory protein
<i>spy0183</i>	2.2	2.5	Putative glycine betaine proline ABC transporter (ATP-binding protein)
<i>spy0184</i>	2.0 (3.4)	2.1 (6.2)	Putative glycine-betaine binding permease protein
<i>spy0528</i>		-2.7	Two-component response regulator VicR
<i>spy0529</i>	-2.5 (-4.9)	(0.8)	Two-component sensor histidine kinase VicK
<i>spy0530</i>	-2.1		VicX of the vicRKX operon
<i>spy0605</i>	-2.0	-1.6	Putative glutathione peroxidase
<i>spy0861</i>	-2.0	-1.8	Mac/IgG endopeptidase
<i>spy1017</i>	1.8	1.7	Hypothetical protein
<i>spy1057</i>	-7.4	-2.3	Hypothetical protein
<i>spy1058</i>	-6.2	-2.8	Putative PTS enzyme II component B
<i>spy1059</i>	-5.9	-4.1	Putative PTS enzyme II component C
<i>spy1060</i>	-5.6 (-7.5)	-4.4 (-4.6)	Putative PTS enzyme II component D
<i>spy1061</i>	-2.5	-1.8	Putative two-component sensor histidine kinase
<i>spy1062</i>	-2.5		Putative two-component response regulator
<i>spy1063</i>	-2.6	-2.0	Putative periplasmic iron-binding protein
<i>spy1181</i>	-3.4	-1.9	Hypothetical protein
<i>spy1183</i>	-2.7	-2.0	Putative decarboxylase, gamma chain
<i>spy1983</i>	-3.8	-2.7	Collagen-like surface protein

*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.

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 (Lukowski *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 pCMV*vicR* 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

Fig. 6. Real-time RT-PCR confirmation of the microarray data for putative VicRK targets *pcsB*, *spy0184* and *spy1060*. TaqMan assays were conducted with total RNA isolated from wt, *vicR::aad*, *vicR::aad/pCMVvicR* and $\Delta vicK$ grown to OD₆₀₀ 0.3. The mean copy number $\pm$ SD of each gene transcript in 30 ng of RNA is shown. The transcript levels are also presented for *emm* as a non-variable control and for *vicK* to examine the polar effect of *vicR* inactivation. The results presented are representative of five triplicate experiments.

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.

Fig. 7. Enhanced OpuA activity and sensitivity to osmotic stress of the *vicR::aad* mutant. wt and *vicR::aad* bacteria were incubated with [¹⁴C]betaine (A) or [³H]proline (B) at room temperature. Data are presented as mean radioactivity $\pm$ SD of a triplicate experiment representative of three experiments. Each measurement used bacteria from 2.5 ml culture at OD₆₀₀ 0.4. (C) Growth of the *vicR::aad* and wt strains in THY with and without added 0.3 M NaCl. wt and *vicR::aad* strains in exponential phase were inoculated into the media, and OD₆₀₀ was monitored at the indicated times.

Similar phenotypes of $\Delta vicK$ 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 ($\Delta vicK$) as an alternative solution. $\Delta vicK$ mutants were easily obtained through allelic exchange, suggesting that there was no selection pressure against $\Delta vicK$ 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 $\Delta vicK$ in non-immune human blood

was 13 % of that of the wt strain. To assess the growth of $\Delta vicK$ in mice in comparison with the wt strain, a mixture of bacteria with a $\Delta vicK$: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 $\Delta vicK$: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 $\Delta vicK$ 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, $\Delta vicK$ did not kill mice under the conditions in which all of the mice infected with the wt strain died (Fig. 8). Thus, $\Delta vicK$ 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* ($\Delta spy1060$) was generated by allelic exchange, and its growth in human blood and virulence in mice were examined. The growth factor of $\Delta spy1060$ was 66 % of that of the wt strain, and the virulence of $\Delta spy1060$ was significantly attenuated in a mouse model of subcutaneous infection ($\Delta spy1060$ 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 [3 H]D-glucose was monitored in the wt, $vicR::aad$, and $\Delta spy1060$. There was no significant difference in [3 H]D-glucose uptake among the strains (data not shown), suggesting that SPy1058–1060 is not a major transporter for glucose.

Fig. 8. Attenuation of GAS virulence by *vicK* and *spy1060* deletion. Groups of eight female CD-1 mice were inoculated subcutaneously with 1.1×10^8 c.f.u. wt, 1.5×10^8 c.f.u. $\Delta vicK$ or 1.2×10^8 c.f.u. $\Delta spy1060$, and survival rates were determined daily. P values: wt versus $\Delta vicK$, <0.0001 ; wt versus $\Delta spy1060$, 0.0048.

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::aad*, 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 infection 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*. $\Delta vicK$ could be generated at a frequency comparable to that of obtaining $\Delta spy1060$, suggesting that there was no selection pressure for a secondary mutation during the generation of $\Delta vicK$; however, both $\Delta vicK$ 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 phosphodonors. The latter could be more important than the former in $\Delta vicK$, 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 phosphodonors. *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 $\Delta vicK$ mutant was more attenuated in virulence than *vicR::aad*. The transcription levels of *pcsB*, *spy0184* and *spy1060* in $\Delta vicK$ were very similar to those in *vicR::aad/pCMVvicR* in THY (Fig. 6). In contrast, *vicR::aad/pCMVvicR* was virulent, while $\Delta vicK$ 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 *pcsB*, 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

This work was supported by grants K22 AI057347 and AR42426 from the National Institutes of Health, grants P20 RR-16455 and P20 RR-020185 from the National Center for Research Resources, and the Montana State University Agricultural Experimental Station. We thank M. Behnke and K. McInerney for assistance in DNA microarray analysis.

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