An insert in the covS gene distinguishes a pharyngeal and a blood isolate of *Streptococcus pyogenes* found in the same individual

Alan F. Garcia,1,† Lucienne M. Abe,1,† Guliz Erdem,2 Chari L. Cortez,1 David Kurahara2 and Karen Yamaga1

1University of Hawai‘i, John A. Burns School of Medicine, Department of Tropical Medicine, Medical Microbiology and Pharmacology, Honolulu, HI, USA
2University of Hawai‘i, John A. Burns School of Medicine, Department of Pediatrics, Honolulu, HI, USA

**Expression of the extensive arsenal of virulence factors by *Streptococcus pyogenes*** is controlled by many regulators, of which CovRS is one of the best characterized and can influence ~15% of the genome. Animal models have established that mutants of covRS arise spontaneously in vivo resulting in highly invasive organisms. We analysed a pharyngeal and a blood isolate of *S. pyogenes* recovered from the same individual 13 days apart. The two isolates varied in many phenotypic properties including SpeB production, which were reflected in transcriptomic analyses. PFGE, multilocus sequence typing and partial sequencing of some key genes failed to show any differences except for an 11 bp insert in the covS gene in the blood isolate which caused a premature termination of transcription. Complementation of a fully functional covS gene into the blood isolate resulted in high expression of CovS and expression of speB. These results, showing a pharyngeal and a blood isolate from a single individual differing by a simple insertion, provide evidence for the model that regulatory gene mutations allow *S. pyogenes* to invade different niches in the body.

**INTRODUCTION**

*Streptococcus pyogenes* (a group A streptococcus; GAS) is a globally distributed pathogen capable of producing a broad spectrum of diseases (Cunningham, 2000, 2008; Musser & DeLeo, 2005; Tart et al., 2007). Two sites of primary infection are pharyngeal cells of the throat and the epidermis of the skin, which usually result in the self-limiting disease of pharyngitis and impetigo, respectively. For some GAS infections, however, more severe, life threatening diseases such as sepsicaemia, toxic shock syndrome and necrotizing fasciitis may ensue. Both variations in host responses and the differential expression of the wide arsenal of virulence factors contribute to the different disease outcomes of GAS infections.

Expression of virulence genes is controlled by about 100 stand-alone and at least 13 two-component regulators (Graham et al., 2002; Nagamune et al., 2005). CovRS is the best characterized two-component regulator (Churchward, 2007). Initially described as regulating hyaluronic acid capsule synthesis (Levin & Wessels, 1998), its ability to control multiple virulence genes became appreciated shortly after its identification (Federle et al., 1999). In fact, the CovRS regulon is responsible for controlling ~15% of the GAS genome, including many of the major virulence factors involved with adhesion to host tissues, evasion of the immune system and enzymes that may promote the spread of the bacteria (Graham et al., 2002). Unusual features of the CovRS system include the ability of CovR to repress rather than activate gene expression and to respond to both external environmental and internal metabolic signals (Churchward, 2007).

Most studies evaluating the effect of CovRS on GAS pathogenesis have relied on mouse models. *covR* mutants increased the transcription of virulence factors compared with their isogenic wild-type, resulting in necrotizing lesions in mice (Heath et al., 1999). In mouse models of skin infections, Ravins et al. (2000) noted that a colony of GAS could switch consistently and irreversibly from a poorly encapsulated colony to a highly mucoid one and that the conversion could be mimicked by changes in
Table 1. Primer sets used for sequencing of selected genes

| Gene (size; bp) | Forward sequence (5′–3′) | Reverse sequence (5′–3′) |
|----------------|---------------------------|--------------------------|
| speB<sup>*</sup> (1197) | Set 1  GTGCCTCAGGTTCTGTGTTAAGC<br>Set 2  TAGCTCTTTCAAACCTTTGTGTTAGGG | CAGCTCAAGAGGATGTGTTGCTACTG<br>GAAGAGGTGTTGCTACTTACCCC |
| ropB<sup>*</sup> (843) | Set 1  CCAAATCAAAAAGCTAACACCA<br>Set 2  CTTTCTATGTTTCGGCATTCA | TTGAACCTCTGATAAATACAATACC<br>ACACGTAACGTATTITTTAATACCT |
| ropB–speB intergenic region<sup>*</sup> (940) | Set 1  CAACGGTTTCACCAATTTCC<br>Set 2  GAGCTAGCCTTGCTCTTGTTGT | TCGGTGATAGGTCCACAACA<br>GCCGCAATACGTGTTGCTACT |
| covR<sup>D</sup> (687) | Set 1  GCACTAAAACAAGAATCAGG<br>Set 2  CTGTGTTCTCTTGTTTAAAGG<br>Set 3  GGCAGTTGCTTCTATTTTTC<br>Set 4  GGGTCGTGAAGGGTTAGAAACTG | GCGGTAGTTGCGAAAAGAGACTG<br>GGGTGGTACGGGCCAAACATG<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT |
| sagA/pel<sup>D</sup> (162) | Set 1  GAAGCCGTTGAGACTAATGTTG<br>Set 2  GGCGCTTTTTGAAGCCTTTAC<br>Set 3  GATTCGCGTACAAGGCTCAT<br>Set 4  GATGGTGGCTTGGTTGGCTT | CCCACGATACCTGATCTGGAATATG<br>GCCGCAATACGTGTTGCTACT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT |
| nra<sup>D</sup> (1536) | Set 1  GCACTAAAACAAGAATCAGG<br>Set 2  CTGTGTTCTCTTGTTTAAAGG<br>Set 3  GGCAGTTGCTTCTATTTTTC<br>Set 4  GGGTCGTGAAGGGTTAGAAACTG | GCGGTAGTTGCGAAAAGAGACTG<br>GGGTGGTACGGGCCAAACATG<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT |
| dppA<sup>D</sup> (1629) | Set 1  AGCCGGTGTATTGTTTAAAGG<br>Set 2  GTTAGACGGCTTCTGAGCA<br>Set 3  TGATCTACGCCGAAACCAGA<br>Set 4  CAGAGCAGAAGGAAAGCACCTA | GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT |
| dppB–E<sup>D</sup> (3117) | Set 1  CCTTCGCTCCTCTTTTCTATCG<br>Set 2  CTGAGCAGCCTGCTGAC<br>Set 3  CTCTTCTGCGCCACAACCCT<br>Set 4  GCTCACGCTTTGCGGCTT<br>Set 5  GCCACGCTTTGCGGCTT<br>Set 6  GCTCACGCTTTGCGGCTT<br>Set 7  GTTCTCACTCCAAGCT<br>Set 8  GCCACGCTTTGCGGCTT<br>Set 9  GCACGCTTTGCGGCTT<br>Set 10 CAGTCTACGCAAGGACCT <br>Set 11 AGCTGATGCTTTGCGGCTT | GCTTAGCTAGATACCTGAAAAAG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT<br>CTGCTAGGCTTGGATGATGGA<br>AGGATATCACGTGTTGCTACT<br>GGGATTTAGCTCAAAGATCTG<br>TGAGTGATGTTGGAAGGAGT

A. F. Garcia and others
CovRS. Engleberg et al. (2001) showed that rare spontaneous and more virulent mutants of covRS could be generated by infecting mouse skins. In an elegant study using microarray analyses, Sumby et al. (2006) identified pharyngeal and invasive transcriptome profiles in human M1 isolates. After subcutaneous injection with a mouse-passaged pharyngeal M1 strain, they found isolates recovered from deep tissue had converted to an invasive transcriptome by an insertion of 7 bp into the covS gene. Such a loss of covS gene expression resulted in the shut-off of SpeB production, which in turn allowed the accumulation of plasmin, triggering systemic spread of the organism (Cole et al., 2006).

In this report, we identify a pharyngeal isolate and a blood isolate of S. pyogenes isolated 13 days apart from a single individual that were genetically similar except for an 11 bp insert into the covS gene leading to a covS truncated mutant. The presence of two isolates that differ in the covS gene may have resulted in the invasion of S. pyogenes into the blood. Such insertions have been well documented in mouse models, but to our knowledge, this is the first report suggesting that a pharyngeal isolate may have converted into an invasive blood isolate by a covS insertion in the same individual.

**METHODS**

**Bacterial strains/clinical isolates.** The recovered isolates were part of a current epidemiological study of GAS in Hawai‘i hospitals approved by the Institutional Review Boards (Erdem et al., 2005, 2009). GAS isolates were obtained from a single patient at Kapi‘olani Medical Center on O‘ahu at two different times. The initial isolate (UH322) was from a 9-year-old male who was diagnosed with pharyngitis. After culturing S. pyogenes, he was prescribed penicillin for 10 days but was compliant for only 5 days. On day 13, he was brought to the emergency room after reporting feeling ill for a few days with insect bites and right inguinal lymphadenopathy. The patient was placed on clindamycin and his blood culture revealed S. pyogenes (UH328) identified using standard methodology. A single colony from the pharyngeal culture recovered earlier and a single colony from the blood culture were inoculated into Todd–Hewitt broth (Difco) supplemented with 0.1 % yeast extract (THY) (Difco) and grown overnight (~20 h) at 37 °C with 5 % CO2. Glycerol was added to a final concentration of 15 % and aliquots were frozen at −80 °C (Erdem et al., 2009). All subsequent studies used these stock cultures.

**DNA isolation, PCR and sequencing.** DNA was prepared using the genomic wizard purification kit (Promega) following the manufacturer’s instructions. Isolated DNA was used immediately or stored at −20 °C. PCRs were performed by using either published primer sequences or primers designed using Primer3 (http://frodo.wi.mit.edu; Table 1) and standard protocols. Other primers used included those for speB, sda, rgg, mga and sagA/pel genes from the paper by Virtaneva.

**Table 1. cont.**

| Gene (size; bp) | Forward sequence (5’–3’) | Reverse sequence (5’–3’) |
|----------------|--------------------------|-------------------------|
| Set 12         | CTGATGAGCCGATTTCAGCTTT   | CGGCTAAATTGACTCGTTCTACC |

*Primers designed using the M1 genome (NC_002737).†Primers designed using the M3 genome (NC_004070).
et al. (2005). For sequencing, the PCR products were treated with Exo-SAP (US Bioscience) and heated. The forward or reverse primer (4 pg μl\(^{-1}\)) was added and submitted to the University of Hawai’i biotechnology facility.

**PFGE and multi-locus sequence typing (MLST).** Isolated DNA digested with Smal, SfiI, Apal, SgrI or SacI (New England BioLabs) was analysed by PFGE as described by Svensson et al. (2000). For MLST, the seven housekeeping genes (*gki*, *gtr*, *muri*, *mutS*, *recP*, *xpt* and *yqiL*) were analysed by PCR using the primers and conditions described on the MLST database (http://spyogenes.mlst.net/misc/info.asp).

**Growth kinetics.** Single colonies from blood agar plates were inoculated into THY until stationary phase was reached. One millilitre of culture at OD\(_{600}\) 1.0 (Biomate 3 spectrophotometer; Thermo Electron Corporation) was added to 150 ml THY. Two millilitres of each culture was removed at the indicated times to measure OD\(_{600}\).

**Adhesion and invasion assays.** Assays were adapted from the method of Hagman et al. (1999). Hep-2 cells (3.8 × 10^5 cells per well) in RPMI 1640, 2 mM glutamine and 10% fetal bovine serum (FBS) were seeded into 24-well plates (Costar) on the day prior to the assay. Bacteria were grown to log or stationary phase in THY, harvested by centrifugation, washed twice, resuspended in RPMI containing 1 % FBS and sonicated for 30 s to disrupt chains. After adjusting the bacteria to 1 × 10^8 bacteria ml\(^{-1}\) based on OD\(_{600}\), 1 ml was added to triplicate wells, centrifuged for 10 min at 125 g to accelerate contact and washed three times with PBS before adding 500 μl 0.025 % Triton X to the wells. Bacterial counts were determined by serial dilution and plating on THY agar. Per cent adherence was calculated by dividing the number of bacteria recovered in each well by the starting inoculum and multiplying by 100.

A similar procedure was used for invasion except that after centrifugation to promote contact, the cultures were incubated further for between 30 and 120 min, at which time the medium was removed, the cultures were washed twice and 400 μg gentamicin sulfate ml\(^{-1}\) (Mediatech) in PBS was added. Samples were incubated for 30 min to kill the extracellular bacteria and washed four times before adding 500 μl 0.025 % Triton X in PBS. Per cent internalization was calculated by dividing the number of bacteria recovered in each well by the starting inoculum and multiplying by 100.

**SpeB assays.** Bacterial supernatants were collected every 3 h during growth kinetic experiments as described above. The supernatants were clarified by centrifugation, split into aliquots and stored frozen at −70 °C until tested. SpeB activity was determined according to the methods described in the text.
samples were tested in triplicate for each run. Either published PCR primers (Virtaneva Technologies) and stored at chloride and 100 % isopropyl alcohol (Fisher Scientific), treated with Biotech) and 10 % SDS. RNA was precipitated with 10 M lithium and by adding phenol/chloroform/isoamyl alcohol (25 : 125 : 1; Fisher extracted by vortexing with acid-washed glass beads (Sigma Aldrich) trifuged at 6000 xg (Jouan) for 10 min and washed. Total RNA was extracted by vortexing with acid-washed glass beads (Sigma Aldrich) and by adding phenol/chloroform/isoamyl alcohol (25 : 125 : 1; Fisher Biotech) and 10 % SDS. RNA was precipitated with 10 M lithium chloride and 100 % isopropyl alcohol (Fisher Scientific), treated with DNase (Ambion), quantified on Agilent 2100 Bioanalyzer (Agilent Technologies) and stored at -80 °C. TaqMan PCR assays of the streptokinase (ska) gene were performed with RNA templates to ensure absence of contaminating genomic DNA.

**RNA isolation.** RNA was isolated from early stationary phase cultures (OD$_{600}$ 0.7–1.0) according to the method of Froeliger & Fives-Taylor (1998) with some modifications. Cultures were centrifuged at 6000 g (Jouan) for 10 min and washed. Total RNA was extracted by vortexing with acid-washed glass beads (Sigma Aldrich) and by adding phenol/chloroform/isoamyl alcohol (25 : 125 : 1; Fisher Biotech) and 10 % SDS. RNA was precipitated with 10 M lithium chloride and 100 % isopropyl alcohol (Fisher Scientific), treated with DNase (Ambion), quantified on Agilent 2100 Bioanalyzer (Agilent Technologies) and stored at -80 °C.

**Microarray.** Total RNA from early stationary phases between the two isolates were compared using custom Nimblegen Systems microarray chips. Total RNA, at 1.0 µg µl$^{-1}$ in nuclease-free water, was sent to Nimblegen for labelling, hybridization and detection. The RNA samples were labelled and hybridized onto two separate custom-made microarray chips containing probes for five different fully sequenced S. pyogenes genomes replicated twice [M1 GAS (NC_002737), MGAS10394 (NC_006086), MGAS315 (NC_004070), MGAS8232 (NC_003485) and SSI-1 (NC_004606)]. Microarray analysis was done using GeneSpring 7.0 (Agilent Technologies) on normalized Nimblegen data. Microarray data were filtered on greater than twofold expression and a P value of ≥0.05. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (GEO) and are accessible through GEO series accession number GSE21316 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21316).

**Quantitative RT-PCR (qRT-PCR).** Single-stranded cDNA was synthesized using Superscript III (Invitrogen) according to the manufacturer’s instructions using SYBR green Supremix on an iCycler (both Bio-Rad Laboratories). The cycling parameters for qRT-PCR were 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s using either published PCR primers (Virtaneva et al., 2003) (Table 1). All samples were tested in triplicate for each run.

**Complementation of a functional covS gene in UH328.** The covS complementation plasmid pJRS325 in Escherichia coli Top 10 cells was provided by Dr June Scott (Dalton & Scott, 2004). The plasmid was purified by caesium chloride/ethidium bromide centrifugation according to methods described by Sambrook et al. (1989). Electroporations were done following the method of Simon & Ferretti (1991) with a few modifications. THY broth (20 ml) was inoculated with 0.4 ml of an overnight culture of S. pyogenes and grown at 37 °C for 3 h to OD$_{600}$ 0.2 (4 x 10$^8$ cfu ml$^{-1}$). Cells were harvested by centrifugation and resuspended in 1 ml ice-cold sterile 0.5 M sucrose. Cells were washed with 0.5 M sucrose and pelleted. The cells were then resuspended in 100 µl 0.5 M sucrose, and 1 µg plasmid DNA was mixed with 40 µl cell suspension and transferred to a chilled Gene Pulser cuvette (0.2 electrode gap; Bio-Rad) and exposed to one electric pulse with the Bio-Rad Gene Pulser (peak voltage 2.5 kV; capacitance 25 F; pulse controller 200 Ω). After the electric pulse, the cells were immediately diluted in 1 ml THY and incubated for 2 h at 37 °C to allow for expression; cells were plated on THY agar containing 0.5 µg erythromycin ml$^{-1}$ and grown overnight. A colony was selected, resolated and used for further studies.

**RESULTS**

**Identical emm sequences, PFGE patterns and MLST**

The pharyngeal (UH322) and blood (UH328) isolates were typed as emm 81.0 and had identical sequences over 450 contiguous nucleotides including the first 240 nt. Both isolates shared the same T pattern, 8/B3264, and both were opacity-factor-positive (data not shown). To expand the genetic analysis, PFGE was performed on both isolates using Smal (Fig. 1a) and four other restriction enzymes: SfiI, Apal, SgrAI and SacII (Fig. 1b). The pharyngeal and blood isolates gave identical banding patterns. MLST analysis revealed identical alleles at all seven loci for both isolates. The alleles for loci gki, gtr, murl, mutS, recP, xpt and yqiL were 38, 2, 60, 23, 39 and 2, respectively, giving a profile sequence type 330.
Phenotypic differences between the pharyngeal and blood isolates

Growth kinetics showed that the pharyngeal isolate grew faster and to a higher density than the blood isolate (Fig. 2a). On enhanced blood agar plates (Fig. 2b), the zone of β-haemolysis was larger around UH322 (diameter 0.5 cm) than UH328 (diameter 0.35 cm). These phenotypic differences between the pharyngeal and blood colonies remained stable after multiple in vitro passages. For the pharyngeal (UH322) isolate, adherence and internalization on Hep-2 cells were greater during the exponential phase, whereas for the blood (UH328) isolate, the stationary phase bacteria adhered and internalized better to Hep-2 cells than the exponential phase cells (Fig. 2c, d).

The most notable phenotypic difference was the production of SpeB by the pharyngeal isolate but not by the blood isolate. SpeB activity peaked for the pharyngeal isolate during late-exponential phase, whereas SpeB activity was not found in any of the culture supernatants from the blood isolate (Fig. 3a). Inclusion of 1 μM E64, a cysteine protease inhibitor, abolished SpeB activity of the pharyngeal isolate. Immunoblot of overnight culture supernatants from the pharyngeal and the
blood isolate confirmed that SpeB was secreted by the pharyngeal isolate and not by the blood isolate (Fig. 3b) whereas streptolysin O was secreted only by the blood isolate (Fig. 3c).

**Transcription analysis comparing the pharyngeal and the blood isolate**

The transcripts that were upregulated in the blood isolate compared with the pharyngeal isolate were determined...
using parametric t-test of transcripts differentially expressed by twofold with a P value <0.05. The number of transcripts that differed between UH322 and UH328 depended on the origin of the probe sets. Comparing the blood isolate relative to the pharyngeal isolate, probes generated from NC002737 (M1) resulted in 92 upregulated transcripts (of which 42 were identified) and 23 downregulated transcripts (10 named); probes generated from NC003485 (M18) showed 85 upregulated transcripts (28 named) and 36 downregulated transcripts (9 named); probes from NC004070 (M3) gave 79 upregulated transcripts (36 named) and 32 downregulated transcripts (16 named); and from NC006086 (M6) gave 69 upregulated transcripts (55 named) and 40 downregulated transcripts (30 named). Probes generated from NC004606, another M3 strain, hybridized poorly to the cDNA from our emm 81.0 isolates and were not analysed further. Those genes differing in expression between pharyngeal and blood isolates are depicted in Fig. 4(a–d) (hypothetical genes and those identified with broad functional categories were removed). Transcripts found predominantly in the pharyngeal isolate included speB, mitogenic factor and streptolysin-S-associated genes. Notable transcripts upregulated in the blood isolate were hasA, which encodes an enzyme responsible for hyaluronate capsule formation, to genes involved with sugar metabolism or phosphorylation of sugars, and sagP, a gene known as an anti-tumour protein but which possibly has arginine deaminase activity. The nomenclature of genes found in NC006086 differed dramatically from the other S. pyogenes strains, so comparisons were difficult; however, using this probe set, streptodornase (sda1) was prominent in the pharyngeal isolate, a gene encoded by a prophage that will be discussed below.

qRT-PCR using total RNA from early stationary phase confirmed the differential expression of the pharyngeal and the blood isolates. We focused on the positive gene regulators ropB, mga and sagA and the negative gene regulator covR, known to affect SpeB production, by qRT-PCR. As controls, two virulence factor genes, speB and sda, and two standards, emm and proS, were tested. The most dramatic difference between the blood and pharyngeal isolates was found in the speB transcript: a blood to pharyngeal ratio of 0.04 was found. sda gave a ratio of 0.13 whereas the positive regulators ropB, saga and mga gave ratios of 0.20, 0.27 and 1.1, respectively. covR showed a low but significant reduction in the blood isolate yielding a ratio of 0.67; at this point, we did not evaluate covS. Both emm and proS yielded a ratio of 1.0.

Sequence analysis of selected genes revealed an 11 bp insertion in the blood isolate

We sequenced both stand-alone and two-component regulatory genes that have been identified as affecting speB expression (Nagamune et al., 2005). The sequences of the positive regulators dppA, B, C and E, mga, oppA, B, C, D and F, RopB, sagA/pel were identical in the pharyngeal (UH322) and blood (UH328) isolates (primers given in Table 1). The possibility that speB (1194 nt) and its intergenic promoter region (940 nt) might differ between the two isolates was tested, but the sequence of this gene in the two isolates was identical. The covR sequences of the two isolates were identical, but an 11 bp insert in covS was found in the blood isolate that was absent in the pharyngeal isolate (Fig. 5). This insert was found using two different primer sets for the covS gene, after decreasing the cycle number from 30 to 20, and after using high fidelity Taq polymerase. The insert was present in stock colonies produced directly from the blood agar plate obtained from the clinical laboratory and in colonies passed many times. The 11 bp insertion at nt 103 in the covS gene resulted in a stop codon in the covS open reading frame, causing a deduced truncated CovS polypeptide of 39 aa from the CovS methionine start codon. The insert was not found in four other emm 81.0 isolates (three blood isolates and one from a deep tissue wound) in our collection (data not shown).

Complementation of functional covS into UH328

qRT-PCR of UH322, UH328 complemented with pJRS325 and UH328 was done using RNA isolated from bacteria grown to early (8 h) and late (12 h) stationary phase (Fig. 6a, b). The insertion of a functional covS complemented UH328 and this strain expressed high amounts of CovS that greatly exceeded that of UH322. However, the general profile of the expressed genes tested by qRT-PCR paralleled that of UH322.

DISCUSSION

We have shown that a pharyngeal (UH322) and a blood (UH328) isolate obtained from the same patient 13 days apart were phenotypically diverse but genetically similar, except that the blood isolate had an insertion of 11 bp in its covS gene. This insertion resulted in a stop codon and a truncated deduced CovS peptide of 39 aa. Among the phenotypic differences between the two isolates, the most noteworthy was the loss of SpeB production, but these also included adherence and internalization characteristics, haemolysis pattern and their growth curve kinetics, features similar to covR/S deletion mutants. The microarray data provided evidence that the pharyngeal isolate gave a pattern similar to the M1 pharyngeal transcriptome pattern and the blood isolate gave a profile similar to the invasive transcriptome profile identified by Sumby et al. (2006), most prominent of which was the increase of the speB transcript in the pharyngeal isolate and the decrease in the has gene transcript relative to the blood isolate. These two transcripts could be identified regardless of the probe origin. Genetic analysis established close DNA similarity between the UH332 and UH328 by PFGE and MLST analyses as well as identifying that several regulator genes had identical sequences (Table 1) except for the insertion in the covS gene.
We do not have direct evidence for the *in vivo* conversion of the pharyngeal isolate into the blood isolate. The most compelling circumstantial evidence is the similarity of our findings to those studies performed in animal models. In a mouse model of skin infections, Ravins et al. (2000) reported that a colony of M6 GAS could switch irreversibly to a more mucoid one and that the switch could be duplicated by mutations in covR/S. Spontaneous mutants of covR/S from experimental mouse skin infections (Engleberg et al., 2001) showed that covR mutations were due to single amino acid substitutions, whereas most of the covS changes were due to frameshift or sense mutations. The covS mutation reported in our work resulted in a frameshift and insertion of a stop codon. Sumby et al. (2006) established that after subcutaneous injection with a mouse-passaged pharyngeal M1 strain, isolates could be recovered from internal organs that had converted from a pharyngeal to an invasive transcriptome due to a 7 bp insert into the covS gene.

It is possible that our blood isolate did not derive directly from the pharyngeal isolate, since our patient could have either possessed more than one *emm* 81.0 isolate initially or encountered another *emm* 81.0 isolate shortly after initial infection. The fact that skin lesions were observed on the child upon his second visit might indicate that the skin infection could have been another source of GAS. However, cultures were not taken from the skin and even if proven to be *emm* 81.0 GAS, they could have been a result of self-infection from the original pharyngeal isolate. We recognize the fact that both PFGE and MLST lack sensitivity for closely related isolates compared with SNP analysis or comparative genomic sequencing (Beres et al., 2006). Complete genome sequencing of UH322 and UH328 may provide direct evidence that the pharyngeal isolate converted to the blood isolate if the two genomes prove to differ only in the 11 bp insert. However, the blood isolate may have undergone additional mutations that led to multiple differences between the pharyngeal and blood isolates. We inserted a functional covS gene into UH328 which resulted in an overexpression of CovS relative to UH322. The expression of *speB*, *sagA*, *rgg/ropB*, *dpp*, *emm* and *sda* in UH328 paralleled that of UH322. In future experiments, we hope to inactivate the covS gene in UH322 to determine if the transcriptomic profile switches to that obtained with UH328. Recently, it has been reported that DNase Sda1, a phage-encoded virulence factor, serves as a selective force for covRS mutation (Walker et al., 2007). The pharyngeal isolate mimics the M1 isolate studied by Walker et al. (2007), in that it appears to contain the

---

**Fig. 5.** Nucleic acid sequence alignment of UH322 and UH328 for the covS gene. The nucleotide sequence is shown for UH322 (top line) and UH328 (lower line) starting from the 5’ end. An asterisk indicates an identical nucleotide in UH322 and UH328. Insertion of the 11 bp at nt 103 for UH328 is represented in bold letters. Insertion of the 11 bp sequence at position 103 in UH328 results in a truncated polypeptide of 39 aa. Underlined letters in UH322 indicate the stop codon for UH328.
phage-encoded Sda1 by promoting covRS mutation, which means the bacteria have a selective advantage. The fact that the patient did not fully comply with the antibiotic treatment and returned to the hospital 13 days later gives temporal evidence that the pharyngeal isolate could have mutated to become systemic. Perhaps infecting cynomolgus macaques, shown to be a good model for GAS pharyngitis (Virtaneva et al., 2003, 2005), and subjecting them to partial antibiotic treatment will clarify whether or not the lack of compliance was a contributing selective pressure for the pharyngeal isolate to enter the blood.

Definitive proof that the pharyngeal isolate changed to a blood isolate cannot be easily established with humans. The finding of our two isolates depended on a serendipitous sequence of events that may have included partial compliance with antibiotic therapy. Regardless of the ultimate conclusions based on future studies, the identification of two genetically similar but phenotypically different emm 81.0 isolates in a single patient gives credence to the model of GAS invasiveness that has been proposed based on animal experimentation (Sumby et al., 2006; Tart et al., 2007; Virtaneva et al., 2003). The animal model shows that GAS can acquire mutations that allow it to escape one niche and enter another that greatly enhances its virulence potential. We hope that future characterization of these two isolates will show that they differ only in the 11 bp insert, thereby more firmly establishing that this model of invasiveness occurs in humans.

ACKNOWLEDGEMENTS

We thank Dr Shannon Bennett for help in the sequence alignment, analysis and comparisons, Esther Volper for her expertise in microarray analysis using Gene Spring 7.0. Dr June Scott provided us with the CovS plasmid as well as giving us helpful suggestions for carrying out the complementation experiments. Dr Sean O’Callahan gave us useful advice and reagents for the plasmid purification and complementation experiments. We would also like to thank Dr Edward Kaplan for help in the early phases of this work. Sequencing was done at the Advanced Studies of Genomics, Proteomics and Bioinformatics facility at the University of Hawai’i at Mānoa. Financial support was received from the Hawai’i Community Foundation, the Chun Foundation (438198), the National Center for Research Resources and the National Institutes of Health (P20RR018727). This work was presented in part at the ASM Conference on Functional Genomics and Bioinformatics, Portland, OR, October 2004; at the 16th Lancefield International Symposium on Streptococcal Disease, Cairns, Australia, May 2005; and the ASM General Meeting, Toronto, Canada, May 2007. No potential conflicts of interest are reported.

REFERENCES

Beres, S. B., Richter, E. W., Nagiec, M. J., Sumby, P., Porcella, S. F., DeLeo, F. R. & Musser, J. M. (2006). Molecular genetic anatomy of inter- and intraserotype variation in the human bacterial pathogen group A Streptococcus. Proc Natl Acad Sci U S A 103, 7059–7064.

Churchward, G. (2007). The two faces of Janus: virulence gene regulation by CovR/S in group A streptococci. Mol Microbiol 64, 34–41.

Cole, J. N., McArthur, J. D., McKay, F. C., Sanderson-Smith, M. L., Cork, A. J., Ranson, M., Rohde, M., Itzek, A., Sun, H. & other authors (2006). Trigger for group A streptococcal M1T1 invasive disease. FASEB J 20, 1745–1747.

Cunningham, M. W. (2000). Pathogenesis of group A streptococcal infections. Clin Microbiol Rev 13, 470–511.

Cunningham, M. W. (2008). Pathogenesis of group A streptococcal infections and their sequelae. Adv Exp Med Biol 609, 29–42.

Dalton, T. L. & Scott, J. R. (2004). CovS inactivates CovR and is required for growth under conditions of general stress in Streptococcus pyogenes. J Bacteriol 186, 3928–3937.

Engleberg, N. C., Heath, A., Miller, A., Rivera, C. & DiRita, V. J. (2001). Spontaneous mutations in the CsrS two-component regulatory system of Streptococcus pyogenes result in enhanced virulence in a murine model of skin and soft tissue infection. J Infect Dis 183, 1043–1054.
Erdem, G., Ford, J., Johnson, D., Abe, L., Yamaga, K. & Kaplan, E. (2005). Erythromycin-resistant group A streptococcal isolates collected between 2000 and 2005 in Oahu, Hawaii, and their emm types. J Clin Microbiol 43, 2497–2499.

Erdem, G., Mizumoto, C., Esaki, D., Abe, L., Yamaga, K., Reddy, V. & Effler, P. (2009). Streptococcal emm types in Hawaii: a region with high incidence of acute rheumatic fever. Pediatr Infect Dis J 28, 13–16.

Federle, M. J., McVler, K. S. & Scott, J. R. (1999). A response regulator that represses transcription of several virulence operons in the group A streptococcus. J Bacteriol 181, 3649–3657.

Froeliger, E. H. & Fives-Taylor, P. (1998). Analysis of adherence-associated gene expression in Streptococcus parasanguis: a method for RNA isolation. Methods Cell Sci 20, 143–151.

Graham, M. R., Smoot, L. M., Migliaccio, C. A., Virtaneva, K., Sturdevant, D. E., Porcella, S. F., Federle, M. J., Adams, G. J., Scott, J. R. & Musser, J. M. (2002). Virulence control in group A Streptococcus by a two-component gene regulatory system: global expression profiling and in vivo infection modeling. Proc Natl Acad Sci U S A 99, 13855–13860.

Hagman, M. M., Dale, J. B. & Stevens, D. L. (1999). Comparison of adherence to and penetration of a human laryngeal epithelial cell line by group A streptococci of various M protein types. FEMS Immunol Med Microbiol 23, 195–204.

Heath, A., DiRita, V. J., Barg, N. L. & Engleberg, N. C. (1999). A two-component regulatory system, CsrR–CsrS, represses expression of three Streptococcus pyogenes virulence factors, hyaluronic acid capsule, streptolysin S, and pyrogenic exotoxin B. Infect Immun 67, 5298–5305.

Levin, J. C. & Wessels, M. R. (1998). Identification of csrR/csrS, a genetic locus that regulates hyaluronic acid capsule synthesis in group A Streptococcus. Mol Microbiol 30, 209–219.

Musser, J. M. & DeLeo, F. R. (2005). Toward a genome-wide systems biology analysis of host–pathogen interactions in group A Streptococcus. Am J Pathol 167, 1461–1472.

Nagamune, H., Ohkura, K. & Ohkuni, H. (2005). Molecular basis of group A streptococcal pyrogenic exotoxin B. J Infect Chemother 11, 1–8.

North, M. (1994). Cysteine endopeptidases of parasitic protozoa. In Methods in Enzymology, pp. 523–539. Edited by A. Barrett. San Diego: Academic Press.

Ravins, M., Jaffe, J., Hanksi, E., Shetzigovski, I., Natanson-Yaron, S. & Moses, A. E. (2000). Characterization of a mouse-passaged, highly encapsulated variant of group A streptococcus in in vitro and in vivo studies. J Infect Dis 182, 1702–1711.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Simon, D. & Ferretti, J. J. (1991). Electrotransformation of Streptococcus pyogenes with plasmid and linear DNA. FEMS Microbiol Lett 66, 219–224.

Sumby, P., Whitney, A. R., Graviss, E. A., DeLeo, F. R. & Musser, J. M. (2006). Genome-wide analysis of group A streptococci reveals a mutation that modulates global phenotype and disease specificity. PLoS Pathog 2, e5.

Svensson, M. D., Scaramuzzino, D. A., Sjobring, U., Olsen, A., Frank, C. & Bessen, D. E. (2000). Role for a secreted cysteine proteinase in the establishment of host tissue tropism by group A streptococi. Mol Microbiol 38, 242–253.

Tart, A. H., Walker, M. J. & Musser, J. M. (2007). New understanding of the group A Streptococcus pathogenesis cycle. Trends Microbiol 15, 318–325.

Virtaneva, K., Graham, M. R., Porcella, S. F., Hoe, N. P., Su, H., Graviss, E. A., Gardner, T. J., Allison, J. E., Lemon, W. J. & other authors (2003). Group A Streptococcus gene expression in humans and cynomolgus macaques with acute pharyngitis. Infect Immun 71, 2199–2207.

Virtaneva, K., Porcella, S. F., Graham, M. R., Ireland, R. M., Johnson, C. A., Ricklefs, S. M., Babar, I., Parkins, L. D., Romero, R. A. & other authors (2005). Longitudinal analysis of the group A Streptococcus transcriptome in experimental pharyngitis in cynomolgus macaques. Proc Natl Acad Sci U S A 102, 9014–9019.

Walker, M. J., Hollands, A., Sanderson-Smith, M. L., Cole, J. N., Kirk, J. K., Henningham, A., McArthur, J. D., Dinkla, K., Aziz, R. K. & other authors (2007). DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection. Nat Med 13, 981–985.

Edited by: M. Killian