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

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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.

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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 septicaemia, 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

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Abbreviations: FBS, fetal bovine serum; GAS, group A streptococci; MLST, multilocus sequence typing; qRT-PCR, quantitative RT-PCR; THY, Todd–Hewitt broth supplemented with 0.1 % yeast extract.

The NCBI Gene Expression Omnibus (GEO) series accession number for the microarray data in this publication is GSE21316 (available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21316).

Table 1. Primer sets used for sequencing of selected genes

Gene (size; bp)	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
speB* (1197)		
Set 1	GTGCCTCAGGTTCTGTTCTAACG	CAGCTACAGGATGTGTTGCTACTG
Set 2	TAGTCTTTCAACCCTTTGTTAGGG	GTAAGGAGGTGTGTCCAATCTACC
ropB* (843)		
Set 1	CCAAATCAAAAAGCTAACACCA	TTGAACCTCGATAAATACAATACCC
Set 2	AATCGATTCATTCCCATAATATCT	TGTCAATGTTGATGAGTTTCTCTTT
Set 3	CCTTCTATGTTTCGGCATTCA	ACACACGTAACGTATTTTTAACATCT
ropB–speB* intergenic region (940)		
Set 1	CAACGGTTTCACCAATTTCC	TCGTGATAGGTCCACAACA
Set 2	TCAGCCAGGAAAAAGCCTAC	TCGGCAAATTACTGGGTTAGC
covR† (687)		
Set 1	TTTGACCATAGAGGGCAGAGA	CGGGCAAGTAGTTCTTCAATG
Set 2	GGGTCGTGAAGGGTTAGAAACTG	GCCGCGGAGATAACGAATATAG
Set 3	CCCGTATTCGTGCTATTTTCC	AGGCAATCAGTGTAAAGGCAGAG
covS† (1503)		
Set 1	GAAGCCGTTGAGACTAATGTTG	CCCACGATACCTGATCTTCGATATG
Set 2	GCAGTCGAGTCATTAGGAGTGAG	GCAACCGGTGTTCGTAATTC
Set 3	GGCGCTTTTTGAAGCCTTTAC	CGGGAATACTTCACAGCATTG
Set 4	GATTCGCGTACAAGGCTCAT	CAGTTGTGGCAAAAGACTGC
sagA*/*pel† (162)	GAGCTAGCCTTGTCCTTGTTGT	TGCTAGCAAACTCATTACCC
nra† (1536)		
Set 1	GCACATACAACAGAATCAGG	GCACATACAACAGAATCAGG
Set 2	CTGTATACCTCGGCAAGGAAAAAG	CAATTGTCGGAGAGGAATATCG
Set 3	GGCGATGTTCTTAAATGG	TTGTTTTGTCAAACCTGTTAAGGA
Set 4	TCAGAGGTTGTGATGATGTTCC	GTCATTTGGGGTCCTTCTTTCT
dppA† (1629)		
Set 1	AGCCCTGAGTATCTTCCTGA	GGGATTTCAGTCAACTGTGCTG
Set 2	GTGAGCCTGTTACTGCTGACGA	TTGCTGGATCGCTAGTGACATC
Set 3	TGATCTACGCGACACCAGAACT	CTGCTAGGCTTGGATGATGTGA
Set 4	CAGAGCAAGCCAAAGCCCTA	AAAGGAGCCGAGGAAACGAT
dppB–E† (3117)		
Set 1	CCTCGGCTCCTTTTCTTATCG	GCTGGTACGATAGCATTTCTAAGG
Set 2	CGTTAGCTGATCGGATTAAGCA	TAGACCTGCAAGCACTCCAAAA
Set 3	CTTTACGCAACCTCGCTCCTT	TGAGTGAAGGTGATGGAGAGGTC
Set 4	CCTTTCTGCCGAACAACCTT	GTGGGCTCATCCGCAATAAT
Set 5	GTCAAACATCAGGTGCGCTTAG	GGTTTGTTCAGGATGTTGTTGG
Set 6	AGCACCTGGTCAAATCTTAGGG	GGCAAAGAACTTGCAGACGA
oppA† (1971)		
Set 1	CGCTACCATGAGAGAATGACAA	GGGCTTTGACACCAACTTCTTT
Set 2	TTTGACTAATGGCGGTGGAAC	GGGTCTGAACCATCTGAACCAT
Set 3	GAACAAGCCTGAAAGCTACTGG	CGCTCGGTCAAAAGCAAACT
Set 4	AGACCCTGCACAACAAGATGC	GGTCTCAGCATAGAAGCCTTGG
Set 5	GAAGCTTTGACAGCTGAAGG	CGGCTTTAGTAACTCGTGGAGTG
Set 6	CCAAGATCCACGGACCTACC	ACGATCGCATGATACGGTTT
oppB† (4425)		
Set 1	TCAAGACAAACCTGTCACAGCA	ATCCTGAATCTTCCAAGGGTGA
Set 2	GTAGTGTAACGACAGAAGCAAC	ACCAAGAGGCAAACCAACGATA
Set 3	ACAAGGGCAAGGACGAACAG	CTGGCCTGAAGGTCAACAAGAT
Set 4	GTTTCTCAACAGCGACCATGAC	CTTCACTATCGCGCTCAACAAA
Set 5	TGGTGGTGCAACCTTAACAGAG	GGCTCCTAAACACTACCCCAATG
Set 6	TCCAATGTTTGCCAACTACGAC	GGTTGGTAAACCAATCCCAAAG
Set 7	GCCTACTCCATCCGTGTTCAA	AGCCTGATTCCCCTACAAAAGC
Set 8	CCACTGACCCACGTTCACATAG	AGGGCTGTTGTTGGCTCATC
Set 9	CGGTAGCCAAATCACAGAAGTG	CCTCTGGGTGTAAAAGCCATGT
Set 10	CAGTTGGCAGATGAATCTGGTG	AAACTAGCAGCGGGATCTTGG
Set 11	AGTTGGAGAATCTGGGAGTGGT	TGGATAACCGCAATACGATCTG

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).

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 % CO_2. 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

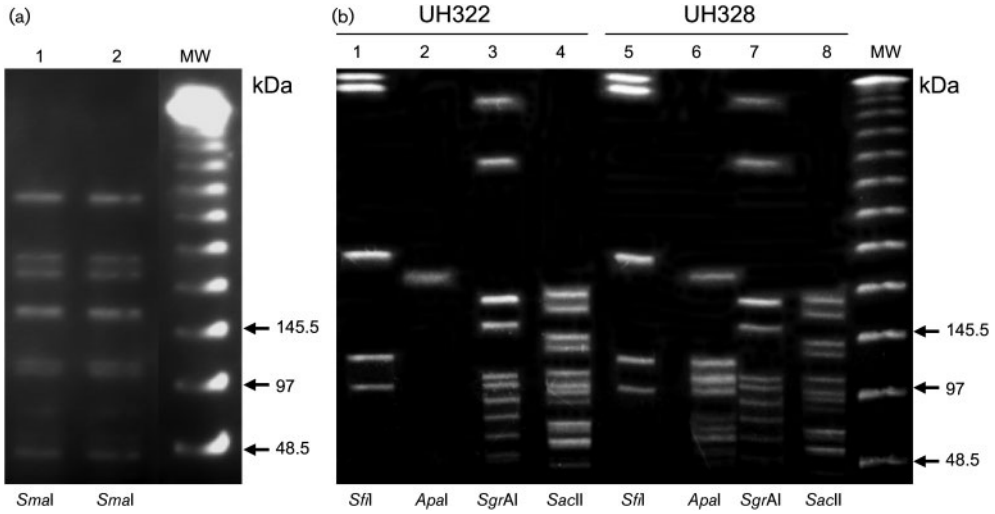


Fig. 1. PFGE patterns of the pharyngeal (UH322) and blood (UH328) isolates. (a) PFGE of *Sma*I-digested DNA of UH322 (lane 1) and UH328 (lane 2). (b) PFGE of *Sfi*I-, *Apa*I-, *Sgr*AI- and *Sac*II-digested DNA of UH322 (lanes 1–4) and UH328 (lanes 5–8). Molecular mass marker (MW) is lambda concatameric DNA marker.

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 *Sma*I, *Sfi*I, *Apa*I, *Sgr*A or *Sac*II (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

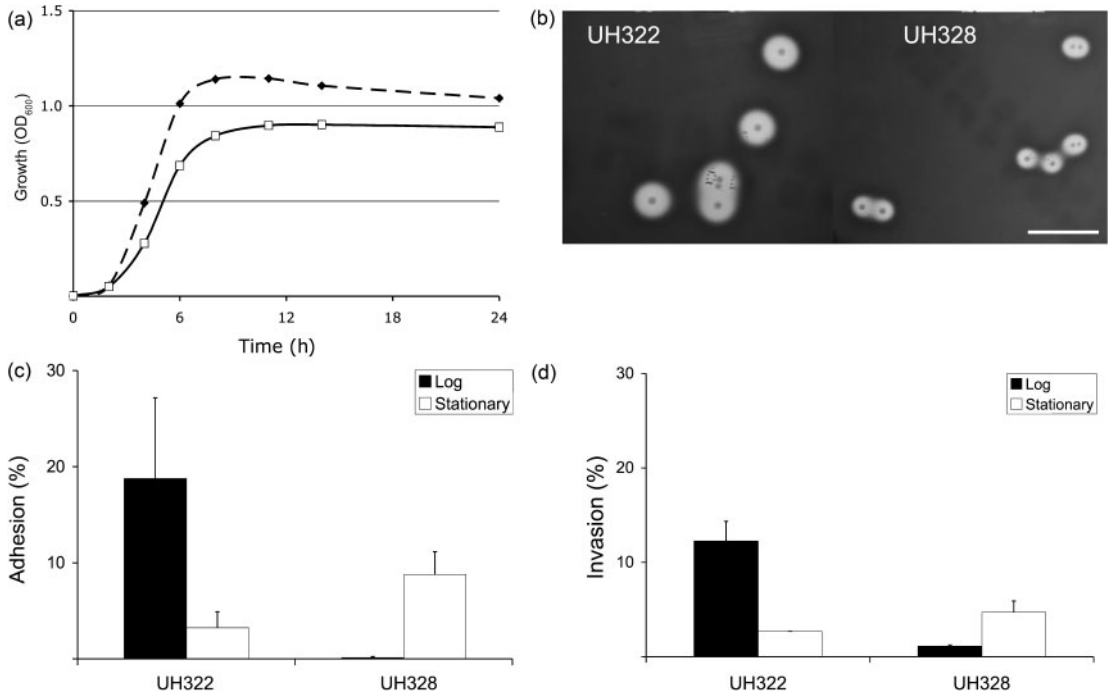


Fig. 2. Phenotypic differences between the pharyngeal and blood isolates. (a) Bacterial growth of UH322 (◆) and UH328 (□) strains was monitored by recording the OD_{600} at various intervals over a 24 h period. The result of a single representative experiment is presented. (b) Haemolysis of UH322 and UH328 isolates on enhanced blood agar plates. The diameter of β-haemolysis surrounding the UH322 isolates measured 0.5 cm (left), whereas the diameter for UH328 isolates measured approximately 0.35 cm (right). Photos were taken under transillumination. Bar, 1 cm. (c) Adherence of stationary and exponential phase cultures of UH322 and UH328 isolates. The bacteria were added to triplicate wells seeded with Hep-2 cells. The per cent adherence was determined by calculating the c.f.u. that adhered to Hep-2 cells divided by the c.f.u. in the original inoculum, and multiplying by 100. The adherence represents mean ± SD of three experiments. (d) Internalization of UH322 and UH328 during stationary and exponential phase cultures. Bacteria were grown to stationary or exponential phase and each was added to triplicate wells of Hep-2 cells, allowed to internalize for 2 h and treated with antibiotics to kill external bacteria. Per cent internalization was determined by dividing the amount of bacteria recovered from lysates of each well by the starting inoculum and multiplying by 100. Values represent the mean ± SD of two experiments.

described by Svensson *et al.* (2000) and North (1995). To ensure cysteine protease activity, 100 μM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E64) (Sigma Aldrich) was added to the culture supernatants to a final concentration of 1 μM before adding the substrate.

For immunoblot assays, SpeB culture supernatants were separated by electrophoresis on a 12 % SDS-PAGE gel and transferred to Biodyne B Membrane (KPL) according to the method by Sambrook *et al.* (1989). Membranes were incubated with appropriately diluted monoclonal rabbit anti-SpeB IgG (Toxin Technologies), goat anti-rabbit conjugated to alkaline phosphatase and alkaline phosphatase substrate according to the manufacturer's instructions (Bio-Rad Laboratories).

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 Bioanalyser (Agilent Technologies) and stored at −80 °C. *Taq*Man PCR assays of the streptokinase (*ska*) gene were performed with RNA templates to ensure absence of contaminating genomic DNA.

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 $\geqslant 0.05$. 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 Supermix 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 choloride/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 (4×10^8 c.f.u. 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, reisolated 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 *Sma*I (Fig. 1a) and four other restriction enzymes: *Sfi*I, *Apa*I, *Sgr*AI and *Sac*II (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*, *murI*, *mutS*, *recP*, *xpt* and *yqiL* were 38, 2, 60, 23, 39 and 2, respectively, giving a profile sequence type 330.

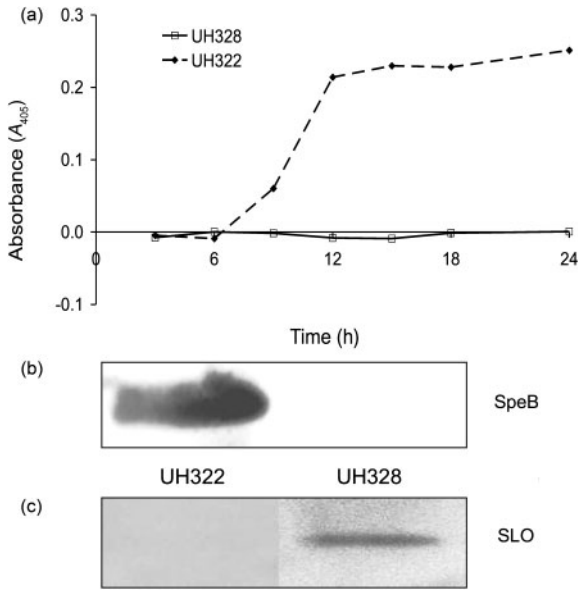
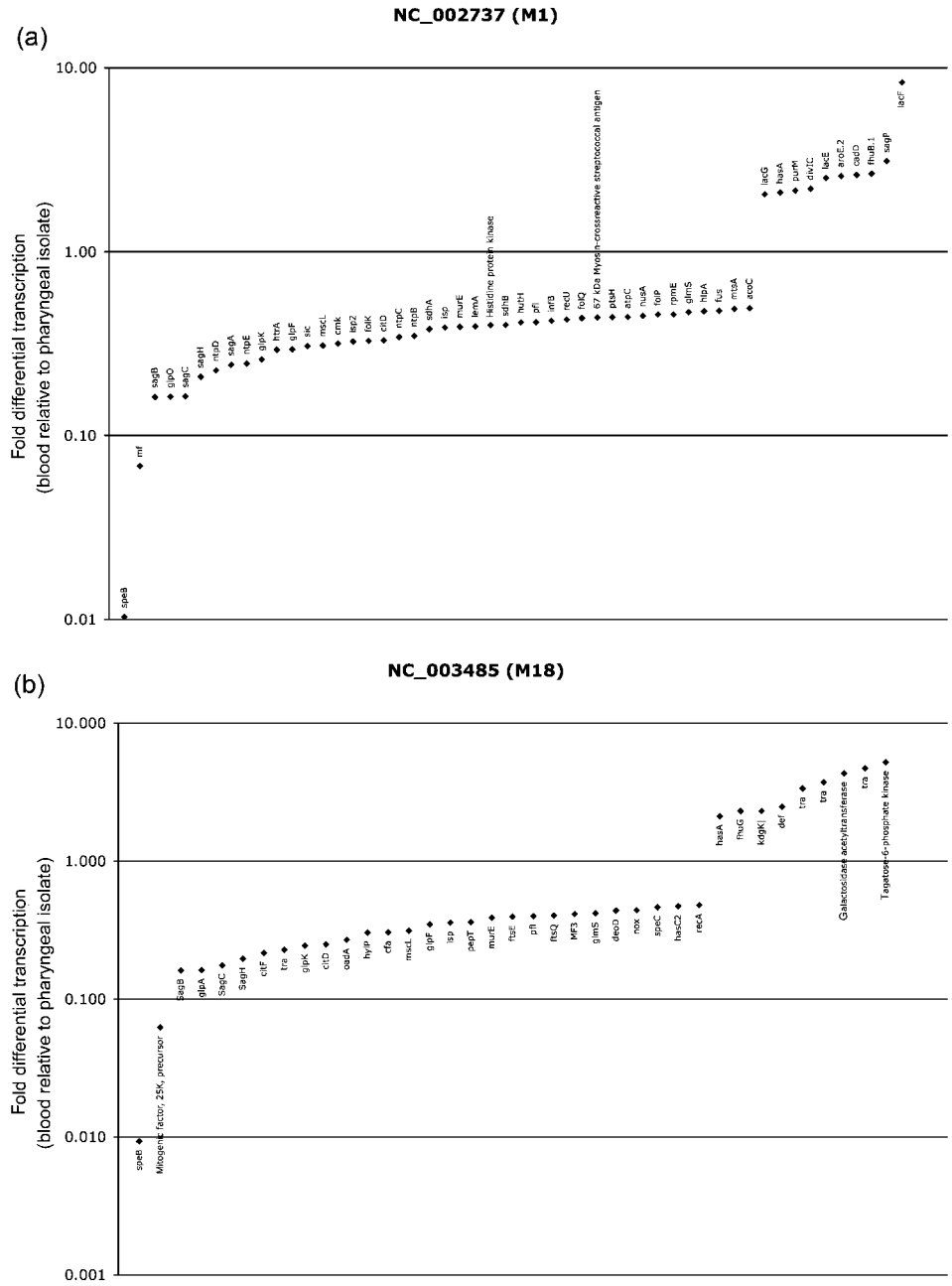


Fig. 3. Functional activity and Western blot analysis. (a) SpeB activity of UH322 and UH328 isolates. Culture supernatants were collected at the indicated times and stored frozen until testing by adding activation buffer and substrates. A_{405} was read to determine substrate cleavage. Results from a single representative experiment are shown. (b, c) Overnight culture supernatants of UH322 and UH328 were resolved on a 12 % SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were probed using rabbit anti-SpeB and anti-Streptolysin-O (SLO), respectively. Bound antibodies were reacted with goat anti-rabbit conjugated to alkaline phosphatase and visualized with alkaline phosphatase substrate.



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

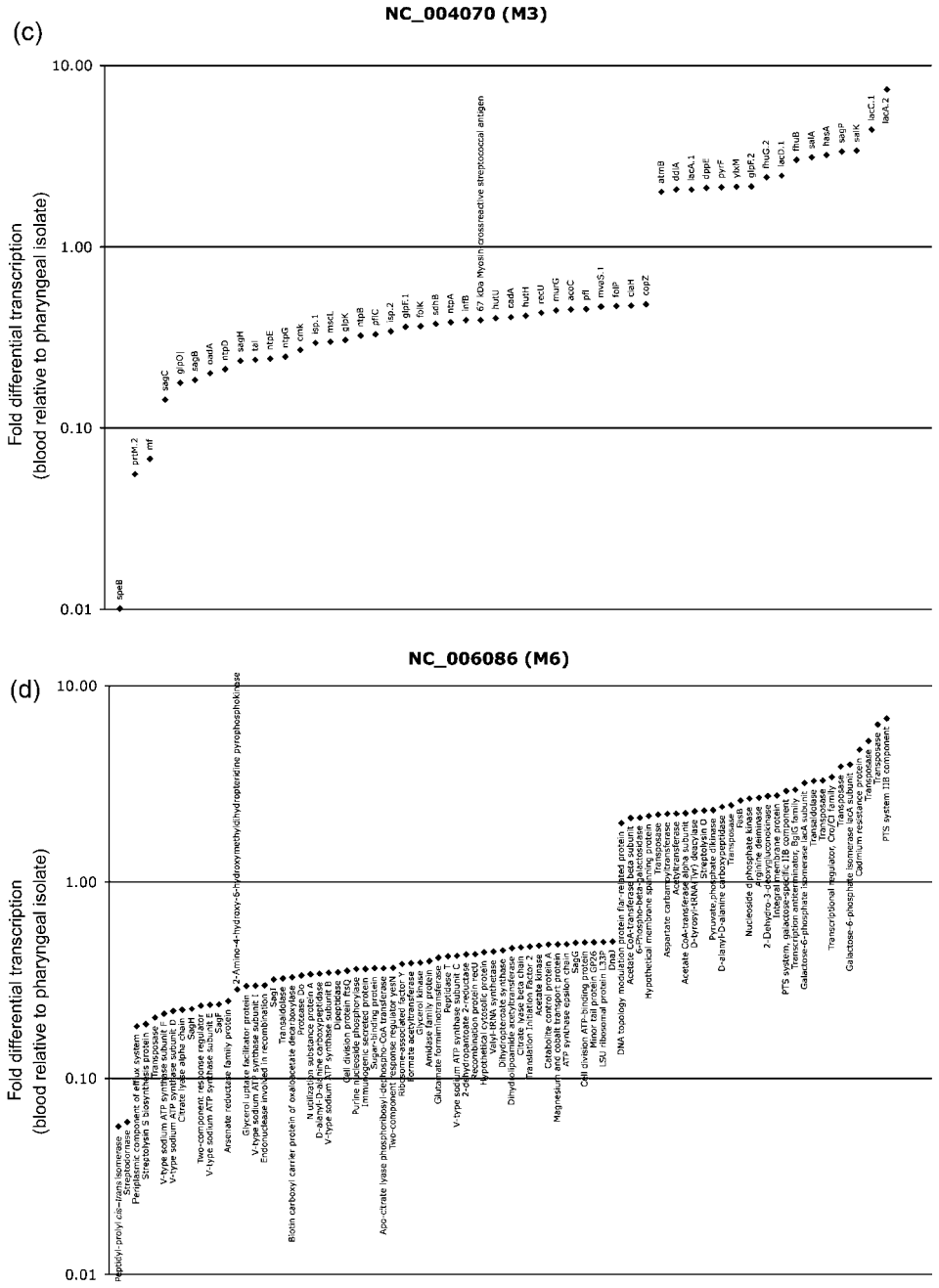


Fig. 4. Transcriptome profile analysis of pharyngeal and blood isolates. Microarrays were done on two separate microarray chips. Expression profiles to five GAS genomes were assessed: NC_002737 (M1), NC_003485 (M18), NC_004070 (M3), NC_006086 (M6) and NC_004606 (M3; not shown). Genes differing in expression by twofold and with a P value $\geqslant 0.05$ between pharyngeal and blood isolates are depicted. Fold transcript differences between the pharyngeal and blood isolates for all known virulence genes and gene regulators are shown. Genes that were expressed at higher levels in the blood isolates are shown above 1.0; genes that were expressed at higher levels in the pharyngeal isolates are shown below 1.0.

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 UH322 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.

```
UH322  5'- ATGGAAAATCAGAAACAAAAACAGAAGAAATATAAAAACTCGTTACCAAAACGACTATCTAATATCTTTTTTGTTCTTTT    80
UH328      ********************************************************************************
           TTTCTGCATTTTCTCTGCCTTT----------ACACTGATTGCCTATAGTTCAACAAACTATTTCTTATTGAAGAAAGA    149
           **********************TCTCTGCCTTT**********************************************
           AAAGCAGTCAGTCTTTCAAGCTGTAAATATTGTTAGAGTTCGTCTTTCTGAGGTGGACTCTAATTTTACATTAGAGAACT    229
           ********************************************************************************
           TAGCAGAAGTTTTGTACAAAAACGATAAAACACATCTGAGAATTGATGACAGAAAGGGCAGTCGAGTCATTAGGAGTGAG    309
           ********************************************************************************
           CGCGATATCACAAATACTCTAGATGCGAATCAAGATATTTATGTCTATAACATTGATAAACAGATGATTTTTACCACAGA    389
           ********************************************************************************
           TAACGAAGAATCATCTCCTGGCTTGCATGGTCCTATCGGTCGGGTGTATCACGACCATATCGAAGATCAGTATCGTGGGT    469
           ********************************************************************************
           TTTCCATGACACAAAAGGTATATTCTAATCGGACTGGAAAATTTGTGGGCTATGTTCAAGTCTTTCATGATTTAGGCAAT    549
           ********************************************************************************
           TATTATGTCATTAGAGCAAGACTGCTGTTTTGGCTACTAGTTGTTGAGTTATTTGGCACAAGCTTAGCCTATTTAATCAT    629
           ********************************************************************************
           CTTAATTACTACGCGGCGCTTTTTGAAGCCTTTACACAATTTACATGAAGTGATGCGTAATATCTCTGAAAATCCTAATA    709
           ********************************************************************************
           ATTTAAACCTGCGCTCAGATATTTCGTCAGGAGATGAAATCGAAGAATTGTCTGTTATCTTTGACAATATGTTAGACAAA    789
           ********************************************************************************
           TTGGAGACACATACTAAGTTGCAATCACGTTTTATTAGTGATGTCAGCCATGAATTACGAACACCGGTTGCGATTATTAA    869
           ********************************************************************************
           AGGGCATATTGGTCTCTTACAACGTTGGGGTAAAGATGACAGTGATATACTTGAAGAAAGCTTGACAGCAACAGCGCATG    949
           ********************************************************************************
           AGGCTGACCGTATGGCAATCATGATTAATGATATGTTAGACATGATTCGCGTACAAGGCTCATTTGAGGGACATCAAAAT    1029
           ********************************************************************************
           GATATGACAGTTTTGGAAGATTCTATTGAAACTGTTGTTGGTAATTTTAGAGTTTTAAGAGAAGATTTTATCTTTACATG    1109
           ********************************************************************************
           GCAGTCAGAAAACCCAAAAACGATAGCCCGTATTTATAAAAATCATTTTGAGCAGGCTTTGATGATTCTTATTGACAATG    1189
           ********************************************************************************
           CTGTGAAGTATTCCCGTAAAGAAAAGAAAATCGCGATTAACCTTTCAGTGACTGGCAAACAAGAAGCTATTGTTAGAGTT    1269
           ********************************************************************************
           CAAGATAAAGGCGAAGGAATTTCTAAAGAAGATATTGAACATATCTTTGAACGCTTTTATAGAACGGATAAATCACGTAA    1349
           ********************************************************************************
           TCGAACAAGTACCCAGGCTGGATTAGGGATTGGCTTGTCTATTCTCAAGCAAATTGTAGATGGGTATCATTTACAGATGA    1429
           ********************************************************************************
           AGGTTGAAAGTGAATTAAATGAAGGTTCAGTGTTTATCTTACATATTCCTTTGGCCCAGTCTAAAGAGAGTTAG -3'     1503
           ********************************************************************************
```

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.

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 frame shift 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

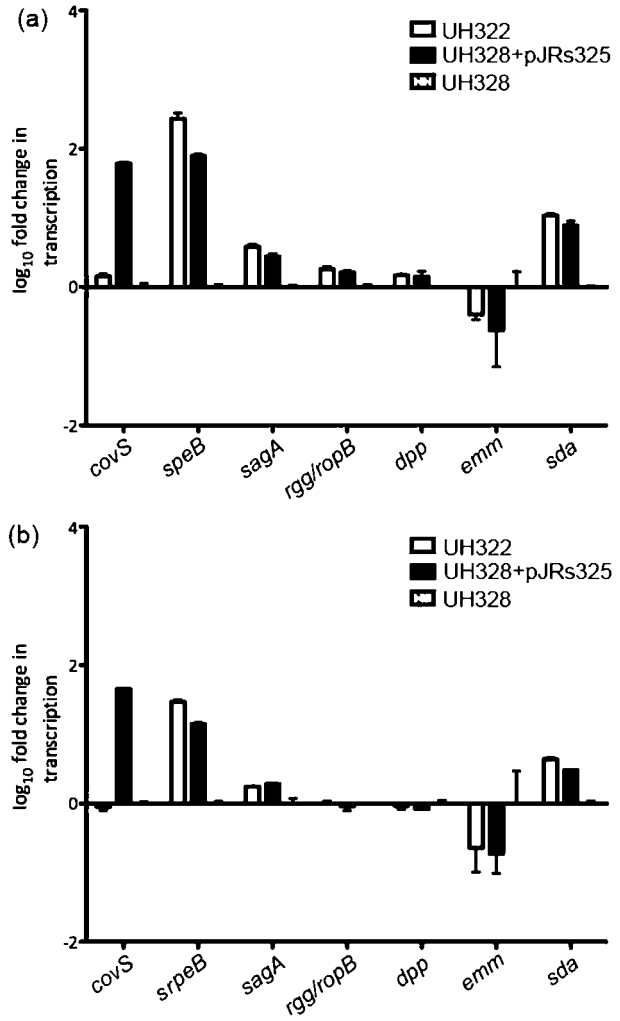


Fig. 6. Quantitative real-time PCR of the pharyngeal and blood isolates, and UH328 complemented with the *covS* plasmid pJRS325. Values are $\log_{10}$ fold changes for each expressed gene relative to *proS* and are expressed as a ratio to UH328 at 8 h (a) and 12 h (b). Bars, SEM of triplicate values.

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.

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